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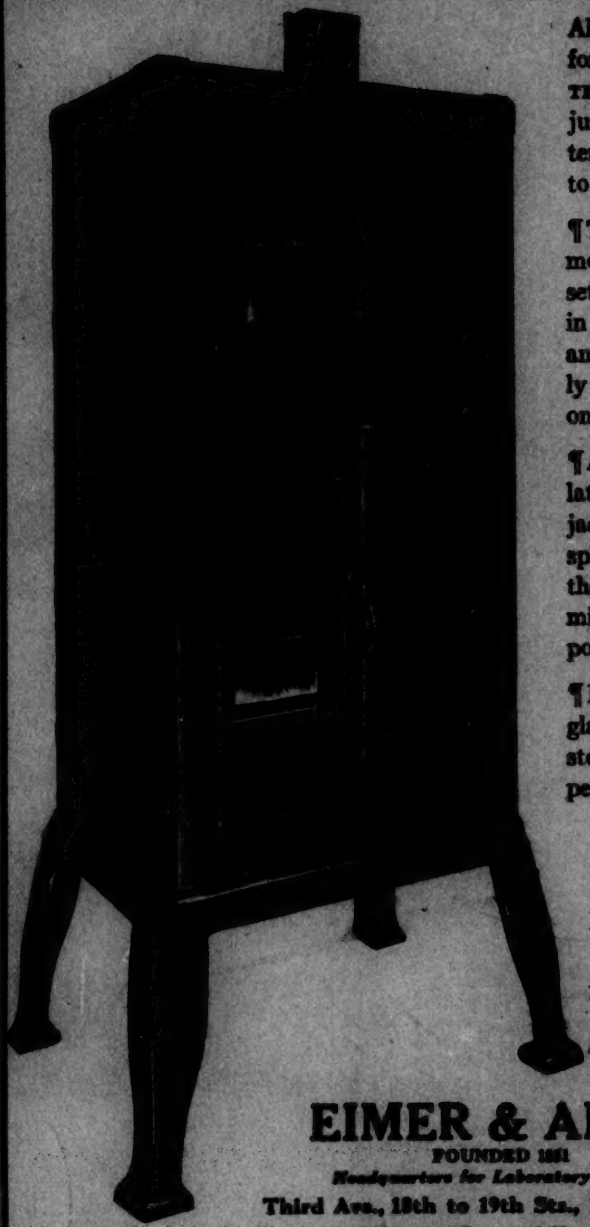

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PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

TWELFTH ANNUAL MEETING.

Minneapolis and Rochester, Minn., December 27–29, 1917.

THE RÔLE OF SOME INORGANIC ELEMENTS IN NUTRITION.

By LAFAYETTE B. MENDEL and THOMAS B. OSBORNE.

(From the Laboratory of the Connecticut Agricultural Experiment Station and the Sheffield Laboratory of Physiological Chemistry in Yale University, New Haven.)

The relative importance of the individual inorganic salts was long ago determined for plants, but hitherto no such definite knowledge of the requirements of animals has been possible owing to the failure of animals to thrive on a diet of purified foodstuffs. Now that the essential factors of a successful dietary have been ascertained it has become possible to prepare rations in which the individual ions are excluded except for the unavoidable small amounts present in the products used to supply the water-soluble vitamine or found as impurities in the other ingredients of the food.

From experiments with such diets it has been demonstrated that calcium and phosphorus are needed in considerable amounts; for with only a trace of either of these elements in the food, rats failed to grow and sooner or later declined. On diets in which only supposedly insignificant quantities of magnesium, sodium, potassium, or chlorine were present growth at a normal or nearly normal rate was made for about 1 year. Where both sodium and potassium were present in the above small amounts no growth occurred.

THE NUTRITIVE PROPERTIES OF KAFIR.

By ALBERT G. HOGAN.

(From the Department of Chemistry, Kansas Agricultural Experiment Station, Manhattan.)

Our investigations of the nutritive properties of kafir were interrupted, and as some time may elapse before this study can be resumed we deem it advisable to take this opportunity of making a preliminary report. Although we have a high degree of

confidence in the correctness of our conclusions, these observations are not to be considered as necessarily final.

When kafir forms the sole diet of white rats, it has about the same nutritional value as corn. Kafir is seriously deficient in calcium, but when this element (Ca lactate) is supplied, the animals grow and attain maturity, though the growth period is considerably prolonged. Growth on kafir and ash mixture is apparently more rapid than on corn and ash mixture.

Females receiving kafir and a salt mixture became pregnant and delivered young, but in no case did these litters survive more than a few days.

The proteins of kafir seemed to have a biological value equal to that of corn in inducing growth.

Kafir is probably deficient in one of the accessory substances. A comparison of the data obtained by feeding kafir and by feeding corn indicates that the fat-soluble A, as described by McCollum, is a limiting factor in kafir.

Our investigations on the nutritive properties of kafirin were brought more nearly to completion and the data has been published in detail.[1] Kafirin, when it forms the sole source of protein in the diet, is apparently inadequate for growth or for the maintenance of young animals (rats). Lysine is the first limiting factor of kafirin, and cystine is the second. The results indicate that lysine is indispensable not only for growth, but also for the maintenance of young animals.

THE RELATION BETWEEN PHOSPHORUS METABOLISM AND THE SECRETION OF MILK FAT.

By EDWARD B. MEIGS and N. R. BLATHERWICK.

(*From the Bureau of Animal Industry, United States Department of Agriculture, Washington.*)

Blood samples have been obtained nearly simultaneously from the jugular and mammary veins of milking cows, and the plasma from such samples has been analyzed for total, lipoid, and inorganic phosphorus, the object being to determine whether the concentrations of these kinds of phosphorus were appreciably

[1] Hogan, A. G., *J. Biol. Chem.*, 1918, xxxiii, 151.

changed during the passage of the blood through the active gland.

In the earlier experiments the cow was disturbed by precautions which were afterward found to be unnecessary, and the results of the experiments differed according as the subjects were much or little disturbed before and during the collection of the mammary blood. When they were much disturbed, the mammary plasma had the same concentration of lipoid phosphorus as the jugular, but a higher concentration of inorganic phosphorus, and a correspondingly higher concentration of total phosphorus. When they were little disturbed, the mammary plasma had a lower concentration of lipoid phosphorus and a higher concentration of inorganic phosphorus than the jugular.

These results are taken to mean that the precursor in plasma of both milk fat and milk phosphorus is either lecithin or some related compound. The ratio phosphorus: fat in lecithin is about 1: 18, whereas it is a out 1: 50 in milk. If, therefore, the mammary gland takes from the plasma enough lecithin or enough of some related phosphatide body to supply its milk with fat, it gets with it more phosphorus than can be used for the milk, and the excess must be returned to the blood. These considerations are taken to explain the back-flow of inorganic phosphorus from the mammary gland to the blood, which occurred in all the experiments. The experiments indicate further that the disturbance of milking animals interferes with the taking up of phosphatide by the mammary gland, but not with the back-flow of inorganic phosphate from the gland to the blood.

THE EFFECT OF COTTONSEED FLOUR ON ANIMALS, WITH PARTICULAR REFERENCE TO THE INORGANIC CONSTITUENTS OF WHITE RATS.

By F. C. COOK.

(*From the Bureau of Chemistry, United States Department of Agriculture, Washington.*)

Six separate groups of animals, each group consisting of twenty-four rats, twelve mice, and six guinea pigs, were fed for periods up to 6 months on (1) 25 per cent cottonseed flour bread, (2) 25 per cent cottonseed flour dough, (3) 40 per cent cottonseed

flour bread, (4) white flour bread, (5) entire wheat bread, and (6) mixed diet.

The exclusive bread diets did not support life, although no pathological lesions were found to be characteristic of any of the diets. 25 per cent cottonseed flour dough supported life longer than 25 per cent cottonseed flour bread. The animals fed entire wheat bread and white bread lived no longer than the animals fed the cottonseed flour breads.

Less nitrogen was required to maintain rats and mice at constant weight when white bread was fed than when entire wheat bread was fed. The largest amount of nitrogen required to maintain constant weight was in the case of the animals fed 40 per cent cottonseed flour bread. These findings are in line with the results reported in the literature for larger animals.

After 6 months, when the experiment closed, the bodies of twenty-four rats, four from each of the six groups, were separately analyzed for inorganic constituents.

The 40 per cent cottonseed flour bread was highest in total ash, P_2O_5, CaO, and MgO of any of the breads fed. The ash constituents were apparently not properly balanced as the bodies of the rats fed on this bread were low in total ash and especially in P_2O_5-free ash. The ratio of MgO to CaO was 1: 15 for the bodies of the rats fed on 40 per cent cottonseed flour bread and 1: 25 for the controls.

The exclusive feeding of cottonseed flour bread apparently caused changes in the ash constituents of the tissues of the rats analyzed. These changes appeared in the results for the rats fed 25 per cent cottonseed flour bread and dough and were marked in the cases of the rats fed 40 per cent cottonseed flour bread.

BORON. ITS EFFECT ON CROPS AND ITS DISTRIBUTION IN PLANTS AND SOILS IN VARIOUS PARTS OF THE UNITED STATES.

By F. C. COOK and J. B. WILSON.

(From the Bureau of Chemistry, United States Department of Agriculture, Washington.)

Borax and colemanite (calcium borate) were found to be effective larvicides for the house-fly.[2] To determine the effect of the

[2] Cook, F. C., Hutchison, R. H., and Scales, F. M., *U. S. Dept. Agric., Bull.* 118, 1914.

boron, added to manure for this purpose, on plant growth a study was made extending over 3 years in which crops were grown in different parts of the country on soil fertilized with manure containing definite amounts of added borax and of added colemanite. In order to test the cumulative effect of the boron the same plants were treated with manure and boron for three seasons. Wheat, rye, oats, beets, tomatoes, cowpeas, lettuce, soy beans, string beans, potatoes, spinach, kale, cabbage, turnips, corn, and peach trees were included in the experiments.

Practically the same amount of boron was absorbed by the plants whether it was added to the soil as soluble borax or as insoluble colemanite. Wheat, oats, and rye absorbed little boron, while leguminous and succulent plants absorbed comparatively large amounts. There was a marked difference in the distribution of the boron in the roots, tops, and fruit of the different plants. Leguminous plants were very sensitive to the boron. At Bethesda, Maryland, 0.0044 per cent H_3BO_3 added as borax and 0.0058 per cent added as colemanite to the upper 6 inches of soil caused no injury to lettuce, spinach, and kale, while at Arlington, Virginia (5 miles distant), the same amounts of boron gave a reduced yield of these crops. A similar difference in the effect of a definite amount of boron on other plants when grown on different soils is indicated. There is a decided difference in soils in rendering the added boron non-toxic, although all soils tested gradually rendered the soluble boron insoluble and non-toxic. In many soils there is a tendency for plants to absorb boron in proportion to the amounts added to the soil while in other soils the same amounts of boron were absorbed irrespective of the amounts added. The absorption and the toxic effect of boron on plants varies with the variety of plants, the solubility of the boron compound, the amount of the boron compound added to the soil, the time elapsing after the compound is mixed with the soil before planting, the amount of rainfall, etc., and finally with the character of the soil to which the boron compound is added.

THE INFLUENCE OF DIET ON THE URINARY CALCIUM AND MAGNESIUM EXCRETION OF MAN.

By MAURICE H. GIVENS.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

Nelson and Burns[3] found that eight out of twenty-five persons, on random diets, excreted more magnesium than calcium in the urine. If one accepts the generally quoted statement that calcium and magnesium excretion is dependent upon the quality and quantity of nourishment ingested, their findings ought to be corroborated with subjects on diets having a content of magnesium greater than that of calcium. Accordingly nine healthy individuals consumed foods estimated to contain more magnesium than calcium for periods of 6 to 9 days. In only three cases was the urinary magnesium excretion greater than that of calcium and in no one of them was the excess of magnesium over calcium greater than 30 mg. per day. In two of these three individuals the urinary calcium output became greater than the magnesium by the addition to the daily intake of two glasses of milk; in the third case a similar result was obtained with a small dose of calcium lactate. When milk was added to the régime the calcium excretion was increased at once with a slight rise in the magnesium.

ACIDOSIS IN RELATION TO PANCREATIC DIABETES.

By V. W. JENSEN.

(From the Northwestern University Medical School, Chicago.)

Elias[4] states that the introduction of acids into rabbits and dogs leads to hyperglycemia and glycosuria. Watanabe[5] in a recent article had occasion to test this out on rabbits and concludes that the results are not at all uniform, and he found that the amount of sugar in the blood was not perceptibly increased. He did not test the urine, but there was no reason to believe that

[3] Nelson, C. F., and Burns, W. E., *J. Biol. Chem.*, 1916–17, xxviii, 237

[4] Elias, H., *Biochem. Z.*, 1913, xlviii, 120.

[5] Watanabe, C. K., *Am. J. Physiol.*, 1917, xlv, 30

there would have been any glycosuria, because of the low amount of sugar in the blood.

The present investigation was commenced without knowledge of this latter work and with the idea of deciding whether or not acid is a cause of glycosuria or a result of a diabetic condition. A great criticism of Elias' work is that the acid he used was too strong, 0.25 N HCl. It is quite possible that his results will hold good, but they are open to the objection that it might have been an indirect action on the pancreas, or a corrosive action on the duodenum.

In the present work, I used 0.1 N HCl, 600 cc. given in 200 cc. doses three times a day. This acid is close to the strength normally found in the dog's stomach. This was tried on two dogs. One dog was rendered glycosuric by removing seven-eighths of the pancreas, and the other highly sensitized by removing six-sevenths of the pancreas. The sensitized dog in no case gave glycosuria by addition of acid, although he could have been rendered highly glycosuric on carbohydrate diet, but was free from it on a meat diet. The glycosuric dog ran on the average 7 gm. of sugar a day on meat diet. During the acid period this amount was doubled. When an equal amount of sodium carbonate was given, the sugar amount dropped to 0.25 gm. per day.

While these experiments are too few in number to give any definite conclusions, it would seem at the present time that acidosis is a result of a glycosuric condition and that after it has developed it acts as a vicious circle and increases the condition. This statement is supported by the fact that when a non-glycosuric sensitized dog is fed a carbohydrate diet, he eliminates 25 gm. of sugar per day, and the acid in urine is doubled, and ammonia excretion is greatly increased.

THE ACTION OF AMMONIUM COMPOUNDS ON PTYALIN.

By ELBERT W. ROCKWOOD.

(From the Department of Chemistry, University of Iowa, Iowa City.)

Digestion was carried on in a medium neutral to litmus. Both salts of inorganic and organic acids were tested. The ammonium salts of the inorganic acids increased the activity of the ptyalin as did also those of the stronger organic acids. In general the

ammonium salts of the strong acids had a greater activating effect than those of the weak acids. To this oxalic acid is an exception, having little or no effect.

LIPEMIA.

By W. R. BLOOR.

(*From the Biochemical Laboratory of Harvard Medical School, Boston.*)

A study was made of the blood lipoids in a case of severe diabetic lipemia where the blood lipoids reached a very high value (over 13 per cent). Analyses were made of blood samples of which the first was taken 36 hours after the last meal and the others at intervals for about 30 days. In the first 20 days of this period no fat was eaten and the total food intake did not exceed 350 calories per day. In the beginning there was marked "diabetic anemia," the blood corpuscle percentage in the first sample being 29 per cent while the normal for this individual was about 42 per cent. During the first 5 days there was considerable acidosis—low CO_2 tension in the alveolar air, high urinary ammonia, and considerable quantities of acetone bodies in the urine—and during this period there was very little absorption of lipoids from the blood; in fact, as a result of the disappearance of the "anemia" and the accompanying concentration of the plasma the lipoid values became higher than at the beginning. The acidosis disappeared during the next 5 day period and with its disappearance there was a rapid lowering of the blood lipoids—about 50 per cent of the total lipoids in the 5 days. Of the lipoids, the fat decreased most rapidly, the lecithin next, and the cholesterol least. The rate of disappearance of the cholesterol was fairly uniform throughout, not being much affected by the presence or absence of acidosis. Changes in the fat and lecithin content of the blood corpuscles were marked throughout, while the cholesterol content remained quite constant, bearing out the earlier findings that the blood corpuscles take an active part in the metabolism of fat and lecithin but not of cholesterol. During the remainder of the period of examination the blood lipoids continued to decrease slowly. The addition of 50 to 60 gm. of fat to the daily diet and the increase of the diet to over 1,000 calories produced only a slight lessening of the rate of decrease.

FURTHER STUDIES IN AUTOLYSIS.

By H. C. BRADLEY.

(*From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.*)

I. Brain.—The autolysis of nervous tissue is accelerated by acids and inhibited by alkali just as are other gland tissues. While the amount of protein present in nervous tissue is very small, the rapidity of its autolysis is about equal to that of liver or kidney. Because of the exceedingly delicate protein framework of the nerve cell, its destruction through the autolytic mechanism may quickly proceed to an irreparable extent, while other organs with their large protein content may undergo large atrophic changes without irreparable damage.

II. Muscle.—Of the three types of muscle tissue, striated autolyzes most slowly and to the smallest extent. Cardiac and smooth muscle autolyze from 50 to 100 per cent faster. All three are found to be accelerated by acidity and inhibited by alkalinity, but to a much less striking extent than the gland tissues previously studied.

III. Colloids.—The work of Ascoli and Izar on the accelerating action of inorganic hydrosols on autolyzing liver was repeated, but not confirmed. Sols of silver, gold, and platinum, prepared by the Bredig method, were found to be wholly inert, neither accelerating nor inhibiting autolysis. Colloidal $Fe(OH)_3$, As_2S_3, and Sb_2S_3 when dialyzed free from electrolytes were found equally inert when added to autolyzing liver pulp. The disagreement between these results and those of the above mentioned authors is so complete that further experiments are under way.

THE INFLUENCE OF MECHANICAL WORK UPON PROTEIN METABOLISM DURING THE HEIGHT OF MEAT DIGESTION IN THE DOG.

By H. V. ATKINSON.

(*From the Physiological Laboratory, Cornell University Medical College, New York City.*)

A dog was given 600 gm. of meat daily in one portion, and the nitrogen eliminated in the urine during the 4th, 5th, and

6th hours was determined. The dog was then caused to run on a treadmill during the 5th hour and it was found that the quantity of nitrogen in the urine was the same as during rest. Therefore, mechanical work has no influence on the rate of absorption per hour or the intensity of protein metabolism per hour in a dog which has been given meat in large quantity.

METABOLIC STUDY OF α-UREIDOISOBUTYLACETIC ACID.

By ALICE ROHDE.

(From the Hooper Foundation, San Francisco.)

The procedure described for the identification of α-ureido-β-phenylpropionic acid in the presence of amino-acids and of urea is found to be applicable to the identification of α-ureido-isobutylacetic acid under similar conditions. This uramino-acid was recovered in considerable quantity from the urine of a cat after slow, continuous injection of a saline solution of the material into the blood stream of the animal.

CHANGES IN THE COMPOSITION OF MUSCLE OF SALMON DURING MIGRATION.

By CARL H. GREENE.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

The muscles of the salmon during the fast of the spawning migration show a great decrease in the amount of fat and protein present, with a corresponding increase in the water content. During the first part of the migration the fat shows the greatest decrease but with the beginning of the disappearance of the fat the protein catabolism becomes marked. On the spawning grounds the fish show great exhaustion of all food reserves in comparison to the initial condition.

The organic extractives as a whole and the extractive nitrogen in particular show a general tendency to increase during the migration fast. There is no apparent relationship in the behavior of the organic and inorganic extractives.

The amino nitrogen in the fat-free tissue increases from 61 to 84 mg. per 100 gm. This increase is most marked calculated on

the basis of the relation between the amino nitrogen and the protein from which it must be formed. Proportionately, the increase is nearly 100 per cent. During the migration the amino-acids are liberated at the expense of an ever increasing wastage of the muscle proteins.

The most significant change lies in the relation of the amino-acids to the water in the muscle. At the beginning the amino nitrogen value calculated per 100 cc. of water increases from 80 to 100 mg. Its concentration then remains unaffected by subsequent changes. With the stimulated protein catabolism the muscles, or more particularly the fluid in the muscles, become saturated with amino nitrogen early in the migration period. This saturation continues through the migration regardless of the great loss of muscle protein, until in the end the exhaustion of the organism proceeds to a degree culminating in death.

The determining factor in the behavior of the amino-acids would seem to be the volume of water present in the muscles and not the protein. This previously undescribed water volume effect is a strong regulating factor and must be added to those suggested by Van Slyke as probably controlling the amino-acid content of the muscles.

THE COMPOSITION OF THE OVARIES OF THE SALMON DURING MIGRATION.

By C. W. GREENE.

(*From the Department of Physiology, University of Missouri, Columbia.*)

Chemical analyses were made of the ovaries of fifteen individual salmon of the species *Oncorhynchus tschawytscha* chosen from five stations of the Columbia River basin during the migration to the spawning grounds. The analytical results, with the exception of the fat, were computed in terms of the fat-free sample, and yield the following comparisons. The neutral fat in the wet sample of ovarian tissue averages 14.15 per cent at the mouth of the river, 17.38 per cent about 210 miles up the river, and drops to 10.38 per cent at the spawning grounds. This fat is a superload in the ovules and cannot be considered a part of the ovarian protoplasm. Leaving the fat out of consideration and calculating on a fat-free or protoplasmic basis, the total

proteins average 29.8 to 31.2 on the lower river, 31.3 in the upper river, and 29.4 at the spawning grounds, a remarkably constant composition. The lipoids decrease from an average of 4.8 at the mouth of the river to 3.3 at the upper river, and 2.85 on the spawning grounds, showing that the percentage of lipoids diminishes with development, at least in this fasting fish. The organic extractives slightly increase during the early stages of migration but decrease during late migration and at spawning. The inorganic salts increase with the migration. Summarizing, it is apparent that the reserve fat of the ovaries decreased with the migration as does also the percentage amount of lipoids present. The organic extractives vary in harmony with similar changes in other tissues of the body though less in amount. The protein content of this growing reproductive tissue remains remarkably constant. In view of the great increase in mass of the ovaries during the migration and fast, from 600 to 1,000 per cent increase, we must look to other tissues for the food supply used in the growth of the ovaries. Analyses already presented show that the muscles of the salmon contribute this material. The muscles decrease in absolute weight and also in percentage of protein and in fat during the migration. The most vital constituent is the protein. The muscle proteins are diminished by 30 per cent during the migration without affecting the physiological or histological characteristics of the tissue. So it is obvious that this great excess of protein must exist in the muscle in the form of stored protein. There is a protein storage in the salmon comparable to the similar storage of fats. This stored protein is available during the migration for the synthesis of new protein tissue in the developing ovaries.

THE PREPARATION OF YEAST NUCLEIC ACID.

By EMIL J. BAUMANN.

(From the Sheffield Laboratory of Physiological Chemistry in Yale University, New Haven, and the Biochemical Department, University of Toronto.)

A new method for the preparation of yeast nucleic acid is suggested based on the facts: (1) that nucleoprotein is a type of acid protein and is separated into nucleic acid and protein by treat-

ment with alkali in the cold; (2) that nucleic acid is soluble in dilute acetic acid; (3) that nucleic acid is precipitated by magnesium sulfate in the presence of hydrochloric acid, as proposed by Slade.

Fresh brewer's yeast is diluted with water and treated with a concentrated solution of sodium hydroxide (100 gm. of alkali per kilo of yeast) and the solution then partially neutralized with hydrochloric acid. The solution is made acid with acetic acid and filtered from the separated protein after the bulk of the precipitate has settled. The filtration is best accomplished with fluted filters or paper pulp. The clear filtrate is treated with 5 per cent of magnesium sulfate and enough hydrochloric acid to cause the nucleic acid to flock out. The precipitated material is the monomagnesium salt and may be dried after washing with alcohol and ether. Yield: 4–7.5 gm. per kilo of yeast.

The analyses for N, Mg, and P are lower than the calculated figures, but the ratios of the three elements correspond accurately with those for monomagnesium nucleate.

The method is applicable to animal nucleic acid with slight modifications.

STUDIES IN ENDOGENOUS URIC ACID METABOLISM.

By HOWARD B. LEWIS, MAX S. DUNN, and E. A. DOISY.

(*From the Laboratory of Physiological Chemistry of the University of Illinois, Urbana.*)

The influence of proteins and various protein derivatives upon the hourly uric acid elimination has been studied in the fasting subject on a purine-free low protein diet. Protein (egg white, cottage cheese) caused a slight rise beginning the 2nd hour after ingestion and increasing to a maximum at the 4th hour. Ingestion of equivalent amounts of nitrogen in the form of amino-acids (glycocoll, alanine) resulted in a sharp rise the 2nd hour after ingestion followed by a prompt return to the normal level. Administration of a second portion of glycocoll following the return to normal caused a like increase in the uric acid excretion. The increased uric acid metabolism was not apparently associated with the phenomena of specific dynamic action of the glycocoll, since aspartic and glutamic acids which probably exert no such

action caused increases in the uric acid excretion of even greater magnitude than glycocoll or alanine. Neither urea nor ammonium chloride administered in amounts comparable to the amino-acids had any influence on the uric acid output. These results are believed to indicate that the rise in uric acid excretion following ingestion of protein food is not due mainly to the work of the digestive glands but to stimulation of the cellular metabolism by the amino-acids liberated in the digestion of the protein. Creatinine excretion was not affected in any of the experiments. Uric acid was determined colorimetrically according to Benedict and Hitchcock.

ASPECTS OF HIPPURIC ACID CONJUGATION.

By E. LACKNER, A. LEVINSON, and WITHROW MORSE.

(From the Nelson Morris Institute, Chicago.)

The liver is concerned with several types of pairing, yet Salkowski reported that the principal seat of hippuric acid conjugation is resident in the kidney. Salkowski's observations have been repeated by others, and variations in the sweeping statements given by him have had to be made. In the dog, Kingsbury found that there are other organs concerned with this type of pairing. The purpose of the following experiments was first of all to determine whether the liver played a recognizable rôle in hippuric acid synthesis, and, secondly, whether, if this is true, the principle could be utilized in detecting obscure liver involvements in man.

Two male dogs were isolated in metabolism cages and the food, water, and excretions observed over a period of 34 days. The urine was collected in 24 hour quantities. Hepatic involvement was made by the use of hydrazine sulfate, which, according to Underhill and Kleiner and to Gideon Wells, produced liver lesions while leaving the kidneys untouched. The truth of this is vouched for by the chloride figures of the present set of experiments as well as by the evidence of autopsy. Hippuric acid was determined by the Folin-Flanders method; chlorides were determined by the method of Volhard; gasometric amino nitrogen determinations were made upon the hydrolyzed urine to determine the glycocoll participation.

The following table gives the résumé of the experiments:

	gm.
(1) Average normal excretion of hippuric acid nitrogen per 24 hours.	
Dog I	0.029
" II	0.032
(2) Average excretion after the administration of 2 gm. of sodium benzoate by mouth.	
Dog I	0.082
" II	0.069
(3) Average excretion after administration of hydrazine sulfate by mouth.	
Dog I	0.043
" II	0.045
(4) Average after injection subcutaneously of 500 mg. of hydrazine sulfate.	
Dog I	0.026
" II	0.030
(5) Average after following this injection with 2 gm. of sodium benzoate by mouth.	
Dog I	0.015
" II	0.014

Averages of the two dogs.

Experiment 1	0.030
" 2	0.075
" 3	0.044
" 4	0.028
" 5	0.014

From these figures, it is evident that normally, an increase in hippuric acid nitrogen after giving sodium benzoate by mouth occurs; that after giving hydrazine by mouth, the hippuric excretion was not affected; that no effect was observed upon the administration of hydrazine subcutaneously, but that *after hydrazine injection followed by sodium benzoate by mouth, less hippuric acid is excreted.*

The kidneys gave every evidence of being normal, as judged by the chloride excretion day by day and also by the condition of the kidney at autopsy. Professor Oscar T. Schultz, director of the Institute, kindly performed the autopsy and reported that Dog II gave wholly normal histological pictures, while Dog I showed some glomeruli and interstitial capillaries engorged, but no other indication of lesion. The livers, on the other hand, showed marked fatty degeneration with central congestion and central necrosis. It is to be concluded, then, that in the dog

the excretion of hippuric acid varies with the condition of the liver, the kidney remaining constant.

If it is true that, in the mammal, the liver participates to a major degree in hippuric acid pairing, we should be able to recognize the involvement of the liver by this means. Several normal children were studied, sodium benzoate being given by mouth, and the hippuric acid figures compared with those obtained upon days when no benzoate is given. Invariably there is an increase in hippuric acid excretion. In one case where there was extensive liver involvement (child of 11 years, mitral regurgitation, heart decompensated, liver greatly enlarged), no increase in hippuric acid excretion following administration of sodium benzoate was observed.

CONCLUSIONS.

1. In dogs, there is a marked participation of the liver in the pairing of hippuric acid.

2. This may be determined by administering subcutaneous injections of hydrazine and following these injections by administration of sodium benzoate by mouth, the hydrazine inducing fatty degeneration of the liver, while the kidney remains intact. There is a lessened output of hippuric acid under these conditions.

3. What appears, from a single crucial instance, to be a similar condition in man was observed.

A MODIFICATION OF THE LEWIS-BENEDICT METHOD FOR THE DETERMINATION OF SUGAR IN BLOOD.

By STANLEY R. BENEDICT.

(*From the Department of Chemistry, Cornell Medical College, New York City.*)

A study of the reaction between picric acid and glucose in alkaline solution was undertaken to find conditions under which this reaction will proceed to completion in dilute solution, thus avoiding the necessity of concentrating by boiling, as in the Lewis-Benedict procedure. The results have shown that in no possible concentration of sodium hydroxide is the depth of color directly proportional to the concentration of sugar. This alkali therefore cannot be employed. With increase of concentration of carbonate during the reaction there is increase in depth of color de-

veloped almost indefinitely, but high concentrations of carbonate lead to the occurrence of secondary reactions, so that the color developed is again not directly proportional to the concentration of sugar. Thus the concentration of carbonate cannot be increased to cause the reaction to go to completion in dilute solution. It was found, however, that in high concentration of picric acid (greater than a saturated aqueous solution) the reaction with glucose will be definite and quantitative in dilute solution in the presence of a moderate concentration of sodium carbonate. Details of the method thus developed will be published shortly.

A PRELIMINARY REPORT ON THE URINARY EXCRETION OF SUGAR IN THE NORMAL DOG.

By STANLEY R. BENEDICT and EMIL OSTERBERG.

(*From Department of Chemistry, Cornell Medical College, New York City.*)

The excretion of sugar in the urine of a normal dog upon various diets was studied by means of a new method for determination of sugar in normal urine. The technique will be published in detail shortly. The results of the study showed that sugar excretion in the normal dog bears no relation to urinary volume, but is dependent upon food ingestion and the nature of the diet. After food is eaten the curve of sugar excretion promptly rises, reaching a maximum usually in the 4th to 5th hour after the feeding. The curve then falls quite rapidly, reaching a low level during the early morning hours. The sugar excretion is less on a meat diet than on a diet rich in starch, but the curve of sugar excretion follows the same general curve in both cases.

A NEW FORM OF COLORIMETER.

By JOSEPH C. BOCK and STANLEY R. BENEDICT.

(*From Department of Chemistry, Cornell Medical College, New York City.*)

An accurate colorimeter has been developed in which the place of costly and difficultly obtainable prisms is taken by mirrors. The instrument will be described in detail later.

ADSORPTION PHENOMENA IN COAGULATION.

By G. H. A. CLOWES.

(*From the Gratwick Laboratory, Buffalo.*)

Physical quality and speed of formation of blood clot appear to depend upon the degree of dispersion of reacting colloids and their initial and final solution concentrations. Much previous work on coagulation is invalidated owing to failure to recognize the extent to which individual coagulation factors are influenced by very slight variations in hydrogen ion content of the medium in the absence of the buffer system.

Precipitated or colloidal barium sulfate, calcium oxalate, etc., may be used to fractionate coagulation factors. Prothrombin is strongly adsorbed by these substances. Adsorption is proportional to the degree of dispersion of the adsorbent and is dependent apparently upon presence of unsaturated lipoids like cephalin, thus indicating probable surface rôle of lipoids in coagulation processes and affording a quantitative method of estimating prothrombin and lipoid.

SALT METABOLISM IN DIABETES.

By A. H. BEARD and L. G. ROWNTREE.

(*From the Department of Medicine, University of Minnesota, Minneapolis.*)

An investigation was undertaken to determine the cause of the gain of weight in diabetes during starvation and on low carbohydrate intake. Gains up to 10 and 12 pounds were encountered during periods of low caloric intake. Edema developed in three cases in a series of twenty-five.

It was found that diabetic patients allowed to use table salt *ad libitum* consumed frequently very large quantities, as high as 40 gm. a day in one instance. Salt and fluid balance charts were constructed and correlated with a weight chart. In some cases it was shown that the gain in weight and development of edema were synchronous with, and in all likelihood dependent upon retention of salt and water.

In one case with restricted sodium chloride intake, but taking large quantities of sodium bicarbonate, retention of fluid and

marked gain in weight and edema developed. On withdrawing sodium bicarbonate and allowing sodium chloride loss of weight occurred.

OBSERVATIONS ON THE PERMANGANATE TITRATION OF URIC ACID CONTENT OF BLOOD.

By J. LUCIEN MORRIS.

(From the College of Medicine, University of Illinois, Chicago.)

The determination of the small amounts of uric acid in blood by permanganate titration in acid solution is subject to a relatively large error. This is due to the continued reduction of permanganate after all uric acid has been oxidized. The transitory nature of the pink color end-point gives an error of only 2 to 3 per cent of the 2 mg. of uric acid in the titration following precipitation from urine as zinc urate. The error is 20 to 30 per cent of the 0.2 mg. of uric acid present in the usual quantity of blood taken for analysis. Oxidation of uric acid by permanganate in the slightly alkaline solution of sodium bicarbonate is a much less vigorous reaction and affords an opportunity of measuring the conclusion of the oxidation. The end-point used is the very delicate iodostarch color, which can be applied to the detection of an excess of permanganate because oxidation of potassium iodide does not take place in the weakly alkaline solution until all uric acid has been oxidized.

The precipitated zinc urate and zinc carbonate are dissolved in hydrochloric acid and disodium phosphate is added until all the zinc has been precipitated. To the resulting solution of uric acid in sodium phosphates, with which the zinc phosphate is present as a precipitate, 25 cc. of a saturated solution of sodium bicarbonate, 5 cc. of a 10 per cent potassium iodide solution, and 1 cc. of a 0.5 per cent soluble starch solution are added. 0.002 N permanganate is run in from a burette until the blue color of the iodide of starch appears. Using this procedure, 0.2 mg. of uric acid (the amount found in 20 cc. of blood) can be determined with an accuracy of 5 per cent or better. The results in blood, as well as the application of this new titration to other organic substances, are being studied further.

ALVEOLAR CARBON DIOXIDE OF THE NEW-BORN.

By MAX SEHAN.

METHODS OF DETERMINING HYDROGEN ION CONCENTRATION AND BUFFER VALUE (ALKALINE RESERVE).*

By J. F. McCLENDON.

THE ISOLATION AND IDENTIFICATION OF THE THYROID HORMONE.

By E. C. KENDALL.

A SIMPLE METHOD OF MAKING STALAGMOMETERS.

By A. D. HIRSCHFELDER.

THE FOOD VALUE OF SOME OF THE LESS USED PACIFIC COAST FISH.

By F. W. ALBRO and H. A. MATTILL.

*Published in full in *J. Biol. Chem.*, 1918, xxxiii, 19.

A NEW METHOD FOR THE DETECTION OF OCCULT BLOOD IN STOOLS.

By WILLIAM G. LYLE and LOUIS J. CURTMAN.

(*From the Harriman Research Laboratory, The Roosevelt Hospital, New York.*)

(Received for publication, November 5, 1917.)

The finding of occult blood in stools is of great importance in the diagnosis of ulcer and cancer of the stomach. However, the usual chemical methods are so unreliable in their results that they are at present discredited by many surgeons and internists. A review of the literature of this subject shows not only that the number of reagents proposed is very large, but that there also exists a considerable number of procedures for the use of these reagents. The two most widely used are gum guaiac and benzidine. The phenolphthalein reagent is difficult to prepare and moreover is entirely too sensitive for ordinary use. In a previous paper[1] we have shown how the benzidine reagent may give erroneous results unless great care is exercised in cleaning all the apparatus as well as in the use of reagents of definite concentrations. In the routine examination of stools we have found it difficult to get good controls with benzidine; moreover, it is too sensitive. An alcoholic solution of gum guaiac possesses the following disadvantages: (1) Not being of fixed composition or purity, a solution of definite concentration cannot be readily prepared. (2) Gums from different sources vary greatly in their sensitiveness as well as in the stability of the blue compound formed.[2] (3) For the best results, solutions must be freshly prepared.

[1] Lyle, W. G., Curtman, L. J., and Marshall, J. T. W., *J. Biol. Chem.*, 1914, xix, 445.

[2] In this connection the following experiment will be of interest. Three specimens of gum guaiac purchased from different dealers were ground and separately dissolved in 95 per cent alcohol in the proportion of 1:60. 0.5 cc. of each of these solutions was added to 2 cc. of an acetic acid-ether extract of a stool and treated with perhydrol. The first did not give a positive test, the second gave only a faint test, while the third yielded a good blue

All the above objections can be overcome by the use of guaiaconic acid which is the active agent of the gum. The preliminary work of this investigation was carried out with Merck's guaiaconic acid; but as this reagent was difficult to purchase, and its cost high, we experimented with gum guaiac in the hope of preparing therefrom some substance which in keeping qualities and sensitiveness would be fully equal to the commercial preparation of guaiaconic acid and, moreover, would possess the additional merit of being readily and economically prepared. This we succeeded in doing.

Preparation of the Reagent.

The method of preparation based on a series of preliminary experiments[3] is as follows: 50 gm. of the ground crude gum guaiac were treated in a beaker with 20 gm. of KOH dissolved in 200 cc. of water. After thorough stirring, the mixture was filtered with the aid of suction through cotton spread out in a thin layer in a Buchner funnel. The residue was washed with water until the combined filtrate and washings approximated 1.5 liters. To the diluted KOH solution were added with constant stirring 21 cc. of glacial acetic acid which was run dropwise from a burette. The precipitate was allowed to settle, the supernatant liquid poured off, and the residue washed once with water by decantation. The precipitate was then transferred to a Buchner funnel and dried by suction as much as possible. The precipitate was gently heated (small portions at a time) in an evaporating dish when most of the water separated and was removed by filter paper. After the removal of the water, and while the mass was still plastic, it was drawn out into thin sheets. In this condition the material rapidly hardens and dries in the air. The dried masses were then ground, treated with 300 cc. of hot 95 per cent alcohol and the mixture was thoroughly stirred to prevent the formation of a gummy mass. In a few minutes a dark brown material separated in a flocculent condition. This was filtered

color but of short duration. Under the same conditions, our preparation gave a good blue color which lasted for some time. The experiment was repeated with the same results.

[3] We wish to acknowledge the assistance of Mr. A. G. Wikoff in the preparation of this substance.

off and the alcohol removed from the solution by distillation. The residue in the flask was treated with 20 gm. of KOH dissolved in water, diluted considerably, and precipitated as before with about 20 cc. of glacial acetic acid. The precipitate was filtered off and dried as described above, after which it was ground and kept in a desiccator. The weight of the material finally obtained was 30.3 gm. representing a yield of 60 per cent. The time required to make this preparation was 4 hours, the distillation of the alcohol being the most time-consuming of all the operations.

A solution containing 1 gm. of this preparation in 60 cc. of 95 per cent alcohol was prepared, kept in a glass-stoppered bottle of colorless glass, and its sensitiveness determined from time to time with a standard solution of blood. No appreciable difference in sensitiveness was observed at the end of several weeks. There is therefore no need for freshly preparing the solution in making the test.

Procedure for the Examination of Stools for Occult Blood.

After considerable experimentation, the following procedure was adopted. Approximately 10 gm. of the stool are transferred to a beaker, 25 cc. of distilled water are added, and the mixture is stirred until of uniform consistency. Over a low flame, the mixture is heated with constant stirring to boiling and kept at the boiling temperature for several minutes. After cooling, one-half of the mixture is transferred to a glass-stoppered bottle of 80 cc. capacity, 5 cc. of Merck's reagent glacial acetic acid and 25 cc. of ether are added, and the mixture is thoroughly shaken and allowed to stand for several minutes. In a test-tube, 2 cc. of the ether extract are treated with 0.5 cc. (1 : 60) of the preparation described in this paper and finally one to five drops of 30 per cent Merck's reagent perhydrol are added slowly from a pipette. A decided green, light or dark blue, or purple color indicates the presence of blood in quantity to be of clinical significance. Our experiments confirmed those of Buckmaster who first pointed out that boiling the blood was without influence on the test with guaiaconic acid.

Determination of the Sensitiveness of the Above Procedure.

To this end a series of standard blood solutions was prepared as follows:

Solution A.—1 cc. of freshly drawn human blood was diluted to 100 cc. in a volumetric flask with distilled water. A few drops of toluene were added as a preservative. 1 cc. of this solution contained 0.01 cc. of blood.

Solution B.—1 cc. of Solution A was made up accurately to 10 cc. giving a solution of strength 1 cc. = 0.001 cc. of blood.

Solution C.—1 cc. of Solution A was diluted to 20 cc., yielding a solution of strength 1 cc. = 0.0005 cc. of blood.

Solution D.—1 cc. of Solution A was diluted to 30 cc., giving a solution the strength of which was 1 cc. = 0.0003 cc. of blood.

Stools Employed in the Tests.

The stools used in the following tests were obtained from patients kept on a meat-free and soup-free diet. They were all formed stools and when tested by the procedure already mentioned gave negative results. 10 gm. of a negative stool were weighed in a beaker, about 50 cc. of water added, and thoroughly mixed; the mixture was then made up with water to 100 cc. and again uniformly mixed. Separate 10 cc. portions of this mixture were respectively treated with definite volumes of the above standard blood solutions, thoroughly mixed, and heated to boiling for 10 minutes with occasional stirring on a hot plate. After cooling, the mixtures were stirred for 5 minutes with 3 cc. of glacial acetic acid and 12 cc. of ether. 2 cc. portions of the clear extracts were transferred to test-tubes, and treated with 0.5 cc. of guaiaconic acid solution and five drops of perhydrol. The following results were obtained.

The experiments in Table I were repeated three times, using different negative stools for each series, with the same results. It appears therefore that when 1 gm. is taken for the test our technique is capable of detecting 0.0001 cc. of blood or one part in 10,000.

It may be of interest to note that when 1 cc. of 3 per cent medicinal hydrogen peroxide was used in place of the five drops of 30 per cent perhydrol, very faint and in some cases negative

TABLE I.

10 Cc. Portions Containing 1 Gm. of Stool.

No.	Blood solution.	Amount of blood in 1 gm. of stool.	Test.
		cc.	
1	0.5 cc. Solution A.....	0.005	Blue.
2	0.1 " " A.....	0.001	Greenish blue.
3	1.0 " " B.....	0.001	" "
4	0.5 " " B.....	0.0005	Light green.
5	0.1 " " B.....	0.0001	" "
6	0.1 " " C.....	0.00005	Negative.
7	0.1 " " D.....	0.00003	"
8	No blood.....	0.0	"

tests were obtained in extracts which gave unmistakably positive results with the usual quantity of perhydrol.

To determine whether any advantage was to be gained by boiling the stool with acetic acid before extraction, the following experiments were made. Four portions, of 9 to 10 gm. each, of a negative, formed stool were treated as follows:

(*a*) To one portion 25 cc. of distilled water were added. (*b*) To another portion 25 cc. of water containing 5 cc. of glacial acetic acid were added. (*c*) Same as (*a*) with the addition of 1 cc. of blood Solution A. (*d*) The same as (*b*) with the addition of 1 cc. of blood Solution A. All were boiled for 10 minutes and the regular procedure was applied with the following results:

a.....Negative.
b..... "
c.....Positive.
d..... "

The above experiments were repeated with the same results, showing that the preliminary boiling of the stool with acetic acid and water possesses no advantage over the adopted procedure of boiling first with a little water and subsequently extracting with acetic acid and ether.

An examination of the various foods and pills given to patients in the hospital was made to see if they gave the test for blood. The procedure was the same as that employed in the examination of stools. For analysis about 25 gm. of each food were taken and

sufficient of the pills to make one dose. Negative results were obtained with the following: American store cheese, custard, milk, prunes, oatmeal, farina, wheatena, bread (white), rice, egg, ice cream, condensed milk, compound C pills, A, B, and S, and A, B, and P, and laxative pills. A positive test was given by meat soup.

Application of the Method to Hospital Cases.

The method described in this paper was used in the examination of over 500 stools to be tested for occult blood with satisfactory results. Before carrying out the test, the patient was kept for at least 2 days on a meat-free and soup-free diet. Although a light green color indicated small quantities of blood, for clinical purposes, we chose to report such cases as doubtful and considered only those cases positive in which a dark green or blue color was obtained.

SUMMARY.

A new procedure for the detection of occult blood in stools is proposed, the chief features of which are:

1. The use of a new preparation derived from gum guaiac, which possesses the merit of being both stable and sensitive.
2. Specific directions for the preparation of the ether extract.
3. The use of perhydrol.

The method was applied to the examination of over 500 specimens with satisfactory results.

THE PROTEINS OF COW'S MILK.*

By THOMAS B. OSBORNE and ALFRED J. WAKEMAN.

With the Cooperation of Charles S. Leavenworth and Owen L. Nolan.

(From the Laboratory of the Connecticut Agricultural Experiment Station, New Haven.)

(Received for publication, November 22, 1917.)

Although casein has been the subject of numerous investigations during the past few years, very little attention has been given to the other proteins of milk, notwithstanding the fact that the published data regarding these are comparatively meager. The reason for this is doubtless based on the idea, which formerly seems to have been held generally, that since casein forms about 80 per cent of the total protein in milk, the other proteins have such a subordinate importance in nutrition that a special investigation of them is not important.

Since recent studies of the chemical constitution, as well as of the relative nutritive value of individual proteins, have shown such wide differences between several of them, and especially since the heat-coagulable protein of milk, the so called lactalbumin, has appeared to be preeminently adapted to the nutritional requirements of growing animals, it has seemed worth while to learn more than is now known respecting these proteins. This has furthermore become important in view of the now generally recognized fact that milk contains among its water-soluble constituents the so called vitamines, which are essential for the growth of young, as well as for the continued maintenance of adult animals.

In all attempts to discover the nature of the water-soluble vitamine in milk it is essential to know the properties and pro-

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

portion of protein which remains in solution after removing casein and the heat-coagulable proteins, for until we know how to remove this we cannot expect to deal successfully with the non-protein nitrogenous constituents of milk. We have therefore devoted much time to a painstaking study of this problem.

No attempt will be made to review the literature relating to this subject which has appeared in widely scattered journals, for in most cases the published details are too scanty to permit a critical comparison of the results with one another, or with those which we have obtained. References to the greater part of this literature can be found in the summaries of the published data relating to the chemistry of milk.[1]

EXPERIMENTAL.

4 liters of skimmed milk, obtained in January from a mixed herd of a neighboring dairy, were saturated with ammonium sulfate and the precipitate was allowed to drain over night on a large folded paper. It was then ground in a Nixtamal mill with water, the volume brought to 3 liters, and stirred for some time until all was dissolved except a little fat. The solution was then filtered through a dense felt of paper pulp and the latter washed with water. The filtrate was opalescent, but transparent. The proteins were again separated by saturating with ammonium sulfate, redissolved in 4 liters of water, and the slightly turbid fluid was treated with about 600 cc. of 1 per cent sulfuric acid which caused the casein to separate sharply. After standing on filters over night in a cold room, protected by toluene, the casein contracted to a dense mass and retained relatively little of the solution.

The clear filtrate was gradually treated with ammonia until neutral to litmus, but as no precipitate formed, the absence of unprecipitated casein, of "acid albumin," and also of calcium phosphate was demonstrated. The neutral solution was acidified slightly with sulfuric acid and saturated with ammonium sulfate. The precipitate was pressed on filter paper, dissolved in water,

[1] Raudnitz, R. W., *Ergebn. Physiol.*, 1903, ii, 193. Rosenau, M. J., and others, *Bull. Hyg. Lab., U. S. P. H., No. 41*, 1908, *No. 56*, 1909. Lane-Claypon, J. E., Milk and Its Hygienic Relations, London, 1916.

and the solution filtered through a felt of paper pulp to remove traces of insoluble matter. The filtrate (about 1 liter) was again saturated with ammonium sulfate and the precipitate allowed to drain on filters over night. When dissolved in water, an opalescent, slightly acid, but transparent solution was obtained. A little of this when dropped into much distilled water gave a turbidity which disappeared on stirring, but no degree of dilution could be found at which a distinct separation of globulin took place. After saturating this solution with ammonium sulfate the precipitate was filtered out and pressed on filter paper over night under a heavy weight. By the foregoing treatment the precipitate, which consisted of all of the milk proteins except those removed by acidifying the milk, was quite thoroughly freed from lactose and other non-protein constituents of the milk. The crumbly precipitate was then dissolved in water, and, in order to remove globulin, its solution was saturated with magnesium sulfate. The resulting precipitate, A, was filtered out and treated as will be described on page 11.

Lactalbumin.

A sample of the filtrate from the globulin, which was perfectly neutral to litmus, was diluted with three volumes of water and heated to 90°. A flocculent coagulum separated very slowly. Another sample, when made distinctly acid to litmus with acetic acid, behaved in the same way. The remaining solution was diluted with three volumes of water, made slightly acid to litmus with acetic acid, and heated to boiling. The coagulum was filtered out, washed with boiling water till freed from sulfate, then with dilute, and finally with absolute alcohol, and dried over sulfuric acid. This preparation of coagulated lactalbumin, which had been freed from globulin by saturating its neutral solution with magnesium sulfate, weighed 10.8 gm., contained 6.25 per cent of moisture, and 0.16 per cent of ash. Its composition, ash- and moisture-free, after drying at 110°, was:

Lactalbumin Free from Globulin.

	Coagulated.			Sebelien.[2]
	I	II	Average.	
Carbon	52.61	52.42	52.51	52.91
Hydrogen	7.18	7.01	7.10	7.18
Nitrogen	15.38	15.48	15.43	15.77
Sulfur	1.92		1.92	1.73
Phosphorus	Trace.		Trace.	0.18
Oxygen (by difference)	.....	.....	23.04	
			100.00	

In harmony with the fact that this coagulated preparation of lactalbumin contained only a trace of phosphorus we found that another preparation which had been similarly precipitated by saturation with magnesium sulfate, but had not been coagulated by heat, when extracted with alcohol yielded only an insignificant amount of substance resembling phosphatide, and that this contained only a trace of phosphorus. It is thus evident that the phosphatide, which we previously obtained from the coagulated protein of milk,[3] is not associated with the purified lactalbumin.

The filtrate from our coagulated lactalbumin gave a slight flocculent precipitate with acetic acid and potassium ferrocyanide, showing the presence of a very little still uncoagulated protein. That a very complete separation of globulin from albumin is obtained by the method employed in making this preparation is shown by the results of anaphylaxis experiments made by H. G. Wells with another preparation made in the same way, except that the final precipitation of the albumin was effected by *acidifying* the filtrate from the globulin precipitated with magnesium sulfate, instead of by heating. This precipitate was freed from most of the adhering solution of magnesium sulfate by pressing between layers of filter paper, and was then dried over sulfuric acid. The preparation thus obtained, which still contained much magnesium sulfate, was perfectly soluble in water. Guinea pigs sensitized by this preparation were promptly killed by a

[2] Sebelien, J., *Z. physiol. Chem.*, 1885, ix, 463.

[3] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1915, xxi, 539.

second injection. On the other hand, those sensitized with a preparation of the carefully purified globulin showed no reaction to a subsequent injection of lactalbumin, nor were they protected against a later injection of the globulin, for animals thus treated died within a few minutes.

Assuming that 1 liter of whole milk, containing 3.5 per cent of fat, is equal to 965 cc. of skimmed milk, the 10.11 gm. of ash- and moisture-free coagulated lactalbumin obtained in this experiment from 4 liters of skimmed milk are equal to about 2.4 gm. per liter of the original whole milk. That the actual amount of lactalbumin was somewhat greater than this is indicated by facts discussed later in this paper in connection with the possible presence of proteoses in milk.

Lactoglobulin.

In order to remove completely lactalbumin from precipitate A (page 9), which was produced by saturating the neutral solution with magnesium sulfate, this was dissolved in about 400 cc. of water, the solution again saturated with magnesium sulfate, and the precipitate filtered out and pressed very thoroughly between filter paper. After repeating this process the final precipitate, when dissolved in about 250 cc. of water, yielded a brownish colored, strongly opalescent, but transparent solution which was just perceptibly acid to delicate litmus paper. Dropped into much distilled water a cloud formed suggesting precipitation, but on mixing only a turbidity remained. When 3 cc. of this solution were mixed with 3 cc. of water 4 or 5 drops of 1 per cent acetic acid and 0.5 cc. of 20 per cent sodium chloride solution, a coagulum appeared on heating to 71°, which separated in much finer flocks than those yielded by heating a solution of lactalbumin. After heating at 71–75° for a short time the coagulum was filtered out. The filtrate gave no turbidity on boiling but did give a slight precipitate when saturated with ammonium sulfate.

The main solution was accordingly diluted with water to 600 cc., made distinctly acid to litmus with 3 cc. of 1 per cent acetic acid, and 30 cc. of a 20 per cent sodium chloride solution were added, which caused opacity in thin layers, but produced

no visible particles. On heating to 72° a flocculent coagulum formed which separated so imperfectly that centrifugation was necessary for its removal. The deposit was washed free from sulfate by centrifuging several times with water, then with 50 per cent alcohol, dehydrated with absolute alcohol, extracted with ether, and dried over sulfuric acid. This preparation weighed 2.4 gm. and contained 8.55 per cent of moisture (dried at 110°) and 2.12 per cent of ash. This is equivalent to 0.52 gm. of moisture- and ash-free globulin per liter of whole milk, assuming that 1 liter of whole milk is equivalent to 965 cc. of skimmed milk. Ash- and moisture-free, this preparation had the following composition:

Lactoglobulin Free from Lactalbumin.

	I	II	Average.
Carbon	51.92	51.85	51.88
Hydrogen	7.01	6.88	6.96
Nitrogen	15.47	15.40	15.44
Sulfur	0.86		0.86
Phosphorus	0.24		0.24
Oxygen (by difference)			24.62
			100.00

Since all of the phosphorus contained in this preparation was recovered in the ash, special investigation was required to determine whether this belonged to phosphatides, to contaminating phosphates, or to other phosphorus-containing substances. Accordingly another preparation was made from 6 liters of centrifugated milk in the same way up to the point of final precipitation with magnesium sulfate. When freed from almost all of the adhering solution by pressing with filter paper, it was reduced to a fine powder and digested for a long time with absolute alcohol. Dried over sulfuric acid this preparation weighed 7.72 gm. and contained 7.21 per cent of nitrogen, equal to 3.6 gm. of globulin, the balance being magnesium sulfate and water. The alcoholic extract when evaporated left a residue weighing 0.1960 gm., thus making the total amount of the magnesium sulfate precipitate 7.92 gm. The residue from the alcoholic

extract was treated with ether, which dissolved all but a little finely divided white substance. This latter had the appearance characteristic of the diaminophosphatide which we previously obtained under similar conditions from the mixed phosphatides extracted from the coagulated milk protein.[3] Without filtering, the ether solution was poured into acetone and yielded a precipitate which contained phosphorus equal to about 0.0800 gm. of phosphatide, equivalent to 2.2 per cent of the lactoglobulin as calculated from the nitrogen in the magnesium sulfate precipitate after extraction with alcohol.

The preparation of lactoglobulin coagulated by heat and extracted with alcohol contained 0.24 per cent of phosphorus, which would correspond to about 7 per cent of phosphatide if all of the phosphorus were present in such substances. Owing to the presence of the relatively large amount of magnesium sulfate it was not possible to determine how much of this phosphorus belonged to inorganic phosphates, but the repeated precipitations with ammonium sulfate ought to have removed this completely.

As just noted, the alcoholic extract of the uncoagulated lactoglobulin contained phosphorus equal to 2.2 per cent of the globulin. In this respect a similarity exists with the vitellin from hen's eggs which on treatment with alcohol yields over 20 per cent of substance, much of which is phosphatide, while the thoroughly extracted protein contains about 1 per cent of phosphorus. It is possible that lactoglobulin is a similar lecithalbumin, or is a mixture of proteins, one or more of which belongs to this group. Further investigations are needed before final conclusions can be reached as to the true nature of this protein which, in its solubility relations, has little in common with most other globulins.

These results, together with the fact that the coagulated lactalbumin as well as the albumin fraction after repeated precipitation with magnesium sulfate in the uncoagulated state yielded only a trace of phosphorus, show that phosphatides are associated only with the lactoglobulin.

Preliminary tests showed that the filtrate from the coagulated globulin contained protein which, even after adding more sodium chloride or acetic acid, could not be caused to separate by longer heating. A liberal quantity of alcohol therefore was

added to the filtrate from the coagulated globulin, and the precipitate, which separated after long standing, was filtered out and digested with water. Since the resulting gelatinous mass could not be washed on filter paper it was suspended in about 400 cc. of 90 per cent alcohol, centrifuged, washed with absolute alcohol, and dried over sulfuric acid. This preparation (lactoglobulin?) weighed 0.97 gm., equal to about 0.2 gm. per liter of the original whole milk.

The alcohol which had been in contact with this smaller part of the magnesium sulfate precipitate was evaporated and the residual solution shaken out with ether. The concentrated ethereal extract was poured into acetone, and a small precipitate which had the appearance of a phosphatide was obtained.

Proteoses in Milk.

We have devoted a great deal of time to determine definitely whether or not proteoses are really present in milk, for this question is not only of interest to the physiologist, but has importance in devising methods for future investigations of the non-protein nitrogenous constituents of milk, as well as for making preparations of our so called protein-free milk which shall contain less protein than the product heretofore employed. Since our numerous experiments have yielded no results from which positive conclusions can be drawn only a general account of their outcome need be given here.

By proceeding according to the conventional methods, first removing casein by precipitation with acid, and albumin and globulin by boiling the acid filtrate from the casein, some protein has always been found in solution. Repeated attempts to obtain sufficient quantities of this protein for detailed examination showed that not only its properties, but also its proportion varied greatly in different experiments, and in many ways it differed distinctly from typical proteoses. Our experience has convinced us that the protein thus escaping coagulation is largely derived from some one or other of the milk proteins through the action of the reagents used. That it is not the result of the action of enzymes or of bacteria in the milk seems to be excluded by the fact that centrifugated milk, saturated with ammonium sulfate

within an hour after drawing from the cow, gave a precipitate which, when worked up as rapidly as possible, yielded substantially the same result as did milk 12 hours old when similarly treated. The acidity of the filtrate from casein, precipitated by either hydrochloric, or by acetic acid, in the presence, or absence, of phosphates, or of sulfates is pH = 4.6 [4] so that coagulation in most of our experiments took place at practically the same degree of acidity. At this degree of acidity some alteration of the globulin or albumin may take place during the heating necessary to coagulate these proteins. We have found however, that if fresh milk is heated to boiling before acidifying and casein together with the coagulated proteins is subsequently removed by acidifying and filtering, a small amount of uncoagulated protein still remains in solution.

No method that we have as yet devised has enabled us to obtain evidence that a certain but relatively very small amount of proteose may not be an original constituent of cow's milk. In our last attempt we obtained per liter of milk only 0.2 gm. of protein resembling proteose although mechanical losses were at the most very small. Until the conditions under which coagulation of the globulin and albumin can be quantitatively effected are established we shall not be in a position to determine definitely whether or not milk contains any proteose.

Siegfried's "Nucleon."

Siegfried[5] obtained from the extract of horse muscle a substance which he called *Fleischsäure*. This he believed to occur in combination with phosphoric acid, which compound he first named *Phosphorfleischsäure*. Since *Fleischsäure* appeared to be identical with antipeptone,[6] Siegfried later proposed to call the phosphoric acid combination nucleon[7] on account of the similarity of its products of hydrolysis to those of the nucleins. Nucleon can be precipitated as an iron compound, carniferrin, which contains

[4] Cf. Allemann, O., *Biochem. Z.*, 1912, xlv, 346. Michaelis, L., and Pechstein, H., *ibid.*, 1912, xlvii, 260.

[5] Siegfried, M., *Ber. Sächs. Ges. Wissensch. zu Leipzig, Math. u. phys. Classe*, 1893, xlv, 485.

[6] Siegfried, *Arch. Physiol.*, 1894, 401.

[7] Siegfried, *Ber. chem. Ges.*, 1895, xxviii, 515.

about 30 per cent of iron, is soluble in an excess of alkali, and behaves like a compound of protein with iron.

Siegfried[8] later obtained a product from cow's milk which he considered to be identical with nucleon from muscle extract, except that it yielded fermentation lactic acid instead of paralactic acid, when hydrolyzed with baryta. He obtained this by precipitating casein with acid and the coagulable proteins by boiling. After removing phosphates by adding calcium chloride and ammonia, and neutralizing the filtered solution, carniferrin, the iron compound of nucleon, was precipitated by adding ferric chloride and boiling.

We have confirmed Siegfried's observation by applying the above procedure to milk serum from which the proteins and phosphates had been removed as he directs and much of the lactose by concentration and crystallization. This solution, however, contained a not inconsiderable quantity of proteose-like protein which could be separated by saturation with ammonium sulfate and readily obtained almost free from phosphorus by treating its solution with baryta, removing the latter with sulfuric acid, and precipitating with an excess of alcohol, a procedure substantially like that employed by Siegfried. Practically all of this proteose was included in the precipitate produced by adding ferric chloride and boiling. However, although a considerable amount of ferric chloride could be added to the filtrate from the precipitate produced by saturating with ammonium sulfate before a reaction with ferrocyanide occurred, no nucleon-like precipitate could be obtained in this solution freed from proteose. Although Siegfried makes no statement as to the presence or absence of proteose-like substances in solution after removing coagulable proteins it would appear that he did not realize that such were present. Since he took no step to remove these, our experience leaves little doubt that such protein formed a part of his carniferrin. It is to be noted that the aqueous extract of ox muscle when freed from coagulable proteins by boiling contains a small amount of protein which can be precipitated by saturating with ammonium sulfate.

Our work with milk has convinced us that a part of the phos-

[8] Siegfried, *Z. physiol. Chem.*, 1895–96, xxi, 360.

phorus of the serum is organically combined and that some of this, at least, is precipitated by ferric chloride under the above conditions, for a sample of the carniferrin precipitate which we obtained, when dissolved in cold dilute nitric acid, gradually deposited a voluminous light yellowish precipitate on standing at room temperature with no evidence of the formation of the characteristic yellow ammonium phosphomolybdate, whereas another sample did not give the yellow precipitate till boiled for some minutes and this gradually increased on continued boiling just as if phosphoric acid were being liberated by hydrolysis. That milk serum, freed from calcium phosphate by neutralizing, still contains phosphorus in organic combination is made probable by the fact that when this is acidified and boiled a precipitate of calcium phosphate forms when the solution is again made alkaline.

In view of these facts it is our opinion that the nucleon which Siegfried obtained from milk was probably a mixture of uncoagulable protein and some still unidentified organic substance which yields phosphoric acid on hydrolysis.

An Alcohol-Soluble Protein in Milk.

From the alcoholic washings of the large quantities of casein which have been made in this laboratory we have isolated several hundred grams of a protein which closely resembles gliadin of wheat in its solubility in alcohol of 50 to 80 per cent. Although the actual amount of this protein in milk is extremely small, the large quantity obtained as a by-product from the necessary processes incident to our feeding experiments seemed to justify a special investigation of its physical and chemical peculiarities. The results of this investigation, which will form the subject of a subsequent paper, have shown that this protein is an original constituent of milk and not a derivative of any of the other proteins contained therein. Since preparations have been obtained so far free from each of the other milk proteins that no evidence of such contamination could be detected by the anaphylaxis reaction, we feel justified in regarding it as a new constituent of this important food product.

A NEW HYDROGEN ELECTRODE FOR THE ELECTROMETRIC TITRATION OF THE ALKALINE RESERVE OF BLOOD PLASMA AND OTHER FROTHING FLUIDS.

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Van Slyke and Cullen have devised a practical method for the determination of the bicarbonate reserve of plasma, based on Van Slyke's method for determining total CO_2. The method presented in this paper also determines the bicarbonate, but by titration of the alkali instead of by the determination of the CO_2 of the $NaHCO_3$ molecule.

At present the most accurate method of titrating the blood alkali against acid to a definite end-point is afforded by the gas chain, since the end-points of indicators are rendered indefinite by the proteins present. With the electrometric technique one may choose between two modes of titration, (1) adding a definite amount of acid and measuring the change in pH, or (2) as in ordinary alkalimetry, adding as much acid from a burette as is necessary to obtain a definite pH. Cullen (1917) in a recent paper has used the former method. The latter, however, has the advantage that it permits one to choose an end-point so near the pH of circulating blood that comparatively little of the acid added combines with proteins and phosphates, so that only the bicarbonate is titrated.

In this paper apparatus is described for a convenient electrometric titration by addition of acid until a desired end-point is reached. As end-point we have chosen the pH of water, which is 7.00 at 23°. During the titration the free carbonic acid is reduced to approximately zero by washing out with hydrogen gas. Under these conditions (pH = 7.0, H_2CO_3 concentration = 0) all the

$NaHCO_3$ is changed to NaCl before the end-point is permanently attained; *i.e.*, for pH = 7 the ratio $\frac{H_2CO_3}{NaHCO_3}$ must be about $\frac{1}{7}$. If, by washing out the CO_2 gas, the H_2CO_3 is reduced to an infinitesimal value, the $NaHCO_3$ is also reduced to approximately zero.

The effect of the titration therefore is to add sufficient HCl to change all the $NaHCO_3$ into NaCl. The phosphates of the serum have a negligible effect on the titration. They are normally only about $\frac{1}{30}$ the molecular concentration of the bicarbonate, and change of pH from 7.4 to 7.0 changes only about one-fifth of the phosphate present from $NaHPO_4$ to NaH_2PO_4. The neutralizing power exerted by the serum proteins for the same pH change is calculated by Henderson to be equal to that of about 0.001 N alkali, while the bicarbonate is normally about 30 times as concentrated. Since the effect of the chief known buffers of the serum (aside from bicarbonate), *viz.*, phosphate and proteins, on the titration is calculated at only $\frac{1}{150}$ and $\frac{1}{30}$ respectively of the bicarbonate effect, the assumption appears justified that our titration measures the serum bicarbonate with a very slight error. As a matter of fact, we find that the amounts of acid required in our electrometric titration of normal human serum are equivalent to an alkali concentration of 0.030 N, which corresponds almost exactly to the average bicarbonate concentration of normal plasma (65 volume per cent CO_2 = 0.029 N) found by Van Slyke and Cullen as a result of direct gasometric determination of the CO_2 portion of the bicarbonate molecule.

In titrating plasma, I have used the rotating electrode (McClendon, 1917, *b*) thus avoiding frothing and eliminating the necessity of pumping, but it was necessary to disconnect the electrode every time more acid was added. It seemed necessary, therefore, to devise an electrode into which acid could be run and through which hydrogen (or H_2-CO_2 mixture) could be run without disconnection from the potentiometer.

After some preliminary forms of electrode had been tried, the rotating electrode shown in Fig. 1 was found satisfactory. The electrode vessel is cylindrical (30 × 36 mm.) with an opening 10 mm. in diameter in one end and the other end cemented to a cork pulley with sealing-wax, and the whole mounted in a wooden frame so as to rotate on a horizontal axis. Through the open end

of the cylinder is passed a large glass tube that fits snugly in the hole. Inside the large tube, three small glass tubes and a rubber tube are passed and cemented in with sealing-wax. The rubber

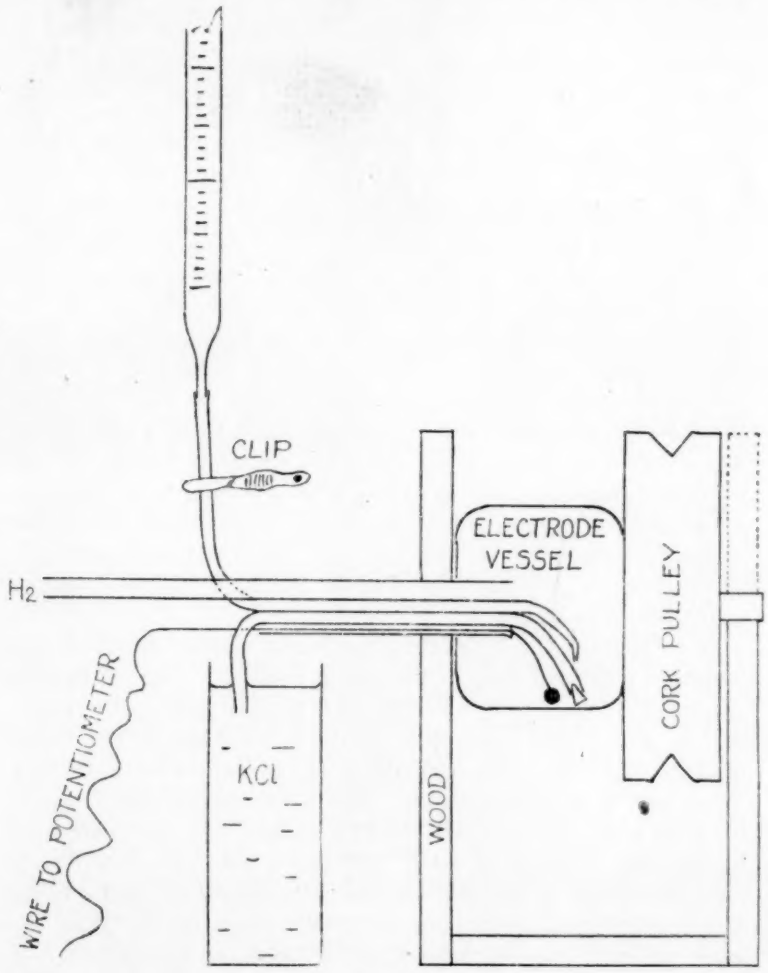

FIG. 1.

tube is 1 mm. bore and filled with a saturated KCl solution. The inner end hangs down in the cylinder and is closed with a bit of match that had been boiled in saturated KCl solution. The

outer end dips into a reservoir of saturated KCl solution connected with the saturated KCl-calomel electrode. The electrode proper is of gold coated with palladium black and connected to a platinum wire fused in one of the glass tubes. The wire is connected with the potentiometer. Lead glass was not used in sealing in the platinum wire owing to danger of reduction of lead thus making the platinum brittle. The 1 mm. glass tube was narrowed at one end so that the platinum wire would just go through. After insertion of the wire, a moment's heating in a tiny blast flame sealed it in the end of the glass tube. About 12 mm. of the platinum wire were allowed to protrude and a gold bead was fused on the end of it. This was accomplished by holding the end of a gold wire in a small Bunsen flame until a bead of the proper size formed, and advancing the end of the platinum wire into the flame until it touched the gold bead, then withdrawing it quickly. The gold bead was hammered to a disc. The finished electrode was put in place so that the gold disc hung down in the electrode vessel. Another glass tube, 1 mm. bore, was drawn to a dropping tip inside the electrode vessel and the outer end connected with a rubber tube of 1 mm. bore closed by a Langenbeck clip and connected with a 1 cc. pipette graduated in hundredths and filled with 0.1 N HCl or NaOH and used as a micro-burette. A glass tube of about 3 mm. bore served for the admission of hydrogen.

In setting up the apparatus, the frame was set in place and weighted with lead so as to remain in position. 1 cc. of plasma was run into the electrode vessel and it was put in place by inserting the axle of the cork pulley in the vertical slot in the frame made to receive it. The cork pulley was connected by means of a string belt to a Tiffany motor. The micro-burette and connecting tube were filled with HCl and the rubber tube was filled with a saturated solution of KCl. The gold disc was coated with palladium black by electrolysis with a 2 volt current and a fairly strong solution of palladium chloride. When this solution became yellow it was evaporated until it appeared light brown in a layer about 2 cm. thick. The electrode was sprayed a moment with distilled water and the 10 mm. glass tube containing the four smaller tubes carefully inserted through the hole in the wooden frame and the hole in the electrode vessel. The 10 mm. tube fits tight in the hole in the frame and, owing to the downward

bending of the small tubes, cannot be shoved straight in but must be started at an angle, and during the process the disc must not be allowed to dry or to touch any solid object. The operation is easier than it may seem. The 3 mm. glass tube is connected with a supply of hydrogen washed with $HgCl_2$, alkaline pyrogallol, and distilled water, and a vigorous stream of the gas passed through the electrode vessel. The Tiffany motor is started and the electrode vessel rotated so as to spread the plasma on its walls, at the same time leaving enough in the bottom to cover the disc. If any portion of the disc dries or gets a clot on it, it must be cleaned and recovered with palladium black. Readings are taken with the potentiometer and acid is run in while the vessel rotates. A 455 ohm galvanometer was used and a balance with the potentiometer took but a few seconds. The palladium black was removed each day by inserting the disc for a moment in aqua regia and spraying with distilled water, and was then deposited again. Since this can be done so quickly and without disconnecting the pipette or hydrogen tube or emptying the rubber tube of a saturated solution of KCl, I have not attempted to determine the length of life of a coating of palladium black. Platinum black has lasted longer in other electrodes but is not so easily removed. The gold disc may be dispensed with, but in order to get surface it is well to use a thick platinum wire, and a thick wire is liable to cause the 1 mm. glass tube to crack. I tried sealing a thick platinum wire into the glass tube with paraffin, and found that it worked for a few determinations. It could be coated with platinum black and cleaned by pulling it out of the paraffin and holding it in a flame. In putting it back, if great care was not used to see that the paraffin seal was perfect, moisture sometimes got into the glass tube and threw out the readings. Some platinic chloride solution that had been used for conductivity electrodes and had perhaps been contaminated with heavy metals, when used to platinize the hydrogen electrode, gave erroneous readings.

The end-point chosen for the titration was the pH of pure water which is about 7.03 at 20°, 7.00 at 22–23°, and 6.95 at 25°. The titrations were made at 23°.

The time required for a titration is not prohibitive for routine work. The collection, oxalation, and centrifugation of the blood under precautions to avoid the loss of CO_2 must be done in any

method. Measuring 1 cc. of the oxalated plasma into the electrode vessel and cleaning and recoating the disc takes but a few moments. If 0.4 cc. of 0.1 N HCl is added at the start the titration back with alkali takes 4 minutes. If the titration is made with HCl the speed with which the pH rises after the addition of acid indicates the amount of acid that it is safe to drop in the next time. The following example shows the time required for titration to the nearest hundredth of a cc. The final two pH readings give a possible method for estimating to thousandths of a cc., but whether such an estimation can be duplicated has not been determined. In some cases a weaker HCl solution was used to ensure more accurate volumetric measurement. In the following table, since seconds are not recorded, in some cases, two operations are placed opposite the same minute.

Hour, p.m.	Burette reading.	pH	Hour, p.m.	Burette reading.	pH
3.49	0.1		3.54		6.96
3.50		7.50	3.55		7.00
3.50	0.2		3.56		7.02
3.51		7.30	3.56	0.31	
3.51	0.3		3.57		6.85
3.52		6.50	4.00		6.87
3.53		6.90	4.05		6.87

The titration in this case took 16 minutes and the alkaline reserve was found to be between 0.03 and 0.031 N. It is not always possible to find the end-point with only four additions of acid, but it is only after the last two burette readings that it is necessary to wait for the definitive pH to be reached. In the following titration six additions of acid were required but the time spent was the same.

Hour, a.m.	Burette reading.	pH	Hour, a.m.	Burette reading.	pH
10.15	0.1		10.21		7:05
10.16		7.4	10.21	0.32	
10.16	0.2		10.22		6.95
10.17		7.20	10.25		7.05
10.17	0.25		10.25	0.33	
10.18		7.10	10.26		6.85
10.18	0.30		10.30		6.85
10.19		6.70			

The whole of 15 minutes need not be spent in titration as it is possible so to regulate the readings that practically all of the last 10 minutes may be spent in doing other things.

In the above titrations, the pH of pure water was taken arbitrarily as the end-point. This method gives at least comparative values and probably is not far from the true end-point, since it should at least determine the bicarbonate. It is impossible to titrate the phosphates, as a pH of about 3 to 4 would be necessary to decompose them, and at this pH the proteins would bind acid. The isoelectric point of serum albumin is at pH = 4.7, and of serum globulin pH = 5.4, and these proteins should not bind acid or alkali at their isoelectric points. These proteins may bind some alkali at pH = 7 and therefore it might seem best to use the mean of their isoelectric points as the end-point for titration. It is necessary, however, to use about a third more acid in order to do this and it seems improbable that the proteins bind a fourth of the alkali at pH = 7. There may be other ampholytes in the plasma with very different isoelectric points, since the concentration of diffusible phosphates is not sufficient to account for this large acid-binding power. According to a rough calculation, the pH of distilled water under an atmosphere containing 5 per cent CO_2 should be near the isoelectric points of serum proteins. It would seem of interest, therefore, to compare the titration of plasma under hydrogen with that under hydrogen containing 5 per cent CO_2. Owing to the fact that no tanks were at hand and my gas mixer holds only 1 liter, I was not sure that equilibrium was reached. A sample of plasma was titrated while H_2 was passing and pH = 7.00 was maintained after 0.33 cc. of acid had been run in. By means of a 3-way cock, H_2 + 5 per cent CO_2 was substituted and the change in pH noted while a liter of the mixture passed through the electrode vessel. The pH gradually fell, finally reaching 6.3. Since this is still far removed from the isoelectric point of the proteins, it seems probable that some other buffer action is present but whether the phosphates could account for this buffer action was not determined.

In the electrometric titration of many solutions the end-point is marked by a more or less distinct angle in the pH curve and it was thought advisable to plot these curves for plasma. The curves plotted by Cullen are almost straight lines and therefore

it would be necessary to remove more of the CO_2 than is accomplished by Cullen's method in order to show an angle or an asymptote. This removal of CO_2 is rapid at first but becomes slower and slower.

In the following table the number of minutes between dropping in the acid and measuring the pH is recorded.

Min.	pH	Burette reading.	Min.	pH	Burette reading.
	8.30		00		0.3
00		0.2	1	7.20	
1	6.70		10	7.35	
5	7.00		15	7.35	
10	7.13		00		0.4
15	7.36		1	6.00	
20	7.50		5	6.05	
25	7.65		10	6.07	
35	7.93		15	6.07	
40	8.02				

The above table merely shows that 40 minutes is not enough for the elimination of the CO_2 when an appreciable amount of the alkali is left in the plasma. The following table shows a similar experiment.

Min.	pH	Burette reading.	Min.	pH	Burette reading.
	8.04		10	7.30	
00		0.10	15	7.31	
1	7.30		00		0.31
2	7.65		1	6.65	
4	7.80		5	6.73	
5	7.85		10	6.73	
10	7.90		00		0.35
15	7.92		1	6.15	
20	7.93		10	6.15	
30	7.94		00		0.40
00		0.20	1	5.45	
1	7.00		5	5.50	
2	7.45		00		0.50
10	7.55		1	4.75	
20	7.56		13	4.75	
00		0.25	00		0.60
1	7.10		1	4.40	
3	7.20		7	4.40	

In the above table the rate of rise of pH with washing out of CO_2 was slower than in the previous experiment, probably due to less rapid flow of hydrogen. Somewhere between pH 5.4 and 4.4 the proteins coagulated, presumably at the isoelectric point. This experiment shows the acid-binding power of the proteins after their isoelectric points have been reached by the slow change in pH on dropping in more acid, the last 0.1 cc. of acid changing the pH only 0.35.

It seems to be impracticable to determine the alkaline side of the titration curve of plasma on titration with acid, and a far better way is the addition of an excess of acid at first and titration with CO_2-free NaOH. In the following experiment, 0.4 cc. of 0.1 N HCl were added to 1 cc. of plasma and titrated with 0.1 N NaOH. The blood had been exposed to air and hence the alkaline reserve of the plasma was not normal.

NaOH	pH	NaOH	pH
cc.		cc.	
0.00	5.40	0.25	9.02
0.05	5.92	0.30	9.43
0.10	6.57	0.35	9.76
0.125	7.00	0.40	10.02
0.15	7.42	0.45	10.24
0.20	8.32	0.50	10.42

On plotting these data a logarithmic titration curve is produced which shows, however, considerable buffer effect. After the alkali is dropped in and well mixed by tilting the apparatus and by the rotation, a fall of potential of less than 2 millivolts is noted. This effect increases as the alkalinity increases and may be due to a slow process of combination of alkali with protein. No argument can be deduced from this curve to show that some other pH than 7 is a more logical end-point for titration. It may be noted that when the alkali equivalent of the acid is dropped in the pH rises to 10. Plasma should reach this pH on driving out the CO_2 with hydrogen, but it would take a long time to accomplish it.

On adding an excess of acid to drive out the CO_2 it is necessary to allow the vessel to rotate and a rapid stream of hydrogen to

flow about 5 minutes or more before beginning to titrate back with NaOH. The following table gives the minutes required, as shown by the rise in pH to an equilibrium.

Min.	pH	Min.	pH
1	4.75	5	5.40
2	5.15	6	5.40
3	5.30	7	5.40
4	5.35	8	5.40

Determinations may be made on 0.1 to 0.5 cc. of plasma using 0.01 N HCl or NaOH. If the plasma does not cover the disc, it must be diluted with 1 per cent KCl solution until it covers the disc.

No undoubted cases of acidosis have yet been studied although I have agreed to do so. Plasma of lowered alkaline reserve was obtained by allowing the CO_2 to escape from the blood before centrifugation. Two such experiments gave the alkaline reserve to equal 0.017 and 0.02 N respectively, but the alkaline reserve before exposure to air was not determined. As may be seen in the above pages, the normal seems to be about 0.03 if pH = 7 is taken as the end-point. I do not, however, know the average normal and merely suppose certain samples to be normal since they were taken from patients without symptoms of acidosis. Van Slyke and Cullen give the average normal chemically bound CO_2 as 65 volume per cent, which I interpret as 0.029 N (since 22.4 liters of CO_2 dissolved in 1 liter of 1 N NaOH would make a 1 N solution of $NaHCO_3$).

It was found that plasma kept for hours at room temperature and then placed in the refrigerator over night presented no difficulties the next day. Plasma kept 36 hours at room temperature poisoned the hydrogen electrode so that no readings could be made. No H_2S was detected in this plasma, and the substance affecting the electrode was not determined.

My thanks are due to members of the staff of the University Hospital for drawing samples of blood.

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APPLICATIONS OF GAS ANALYSIS.

IV. THE HALDANE GAS ANALYZER.

By YANDELL HENDERSON.

(From the Physiological Laboratory, Yale Medical School, New Haven.)

(Received for publication, October 30, 1917.)

This apparatus has such marked advantages for precise analysis of gas mixtures containing less than 30 per cent of oxygen, CO_2, and other absorbable or oxidizable gases that it has become the standard instrument. Nevertheless it has several features in which experience in this laboratory has shown that it may be simplified with advantage. The form of apparatus shown in Fig. 1 is the result of this experience. The advantage of this form is not in its use during analysis,—for this involves no significant change,—but chiefly in the ease with which it can be taken apart and cleaned (a feature in which the usual form of the apparatus is a source of much annoyance and waste of time), and in the lessened liability to "frozen" cocks and breakage. It has a minimum of rubber connections and only one glass stop-cock. (I have also tried out an apparatus with connections of glass tubing of only 0.7 mm. bore, but have found this too small. The usual bore of 1.1 to 1.3 mm. is best.)

The particular features of this form of the apparatus are: (1) a 4-way glass stop-cock shown in Fig. 2; (2) the adjustment of the volume of the control tube by means of a screw pinch-cock on a bit of rubber tubing on the lower end of the tube; (3) two large test-tubes in which the absorbents KOH or NaOH and potassium pyrogallate[1] are placed. The fine adjustment of pressure-volume is made by raising or lowering the test-tube containing the alkali. For the oxygen absorption the test-tube containing the pyrogallate may be raised and lowered, to avoid

[1] Sodium pyrogallate (as described by Shipley, J. W., *J. Am. Chem. Soc.*, 1916, xxxviii, 1687) has not proved satisfactory in this instrument.

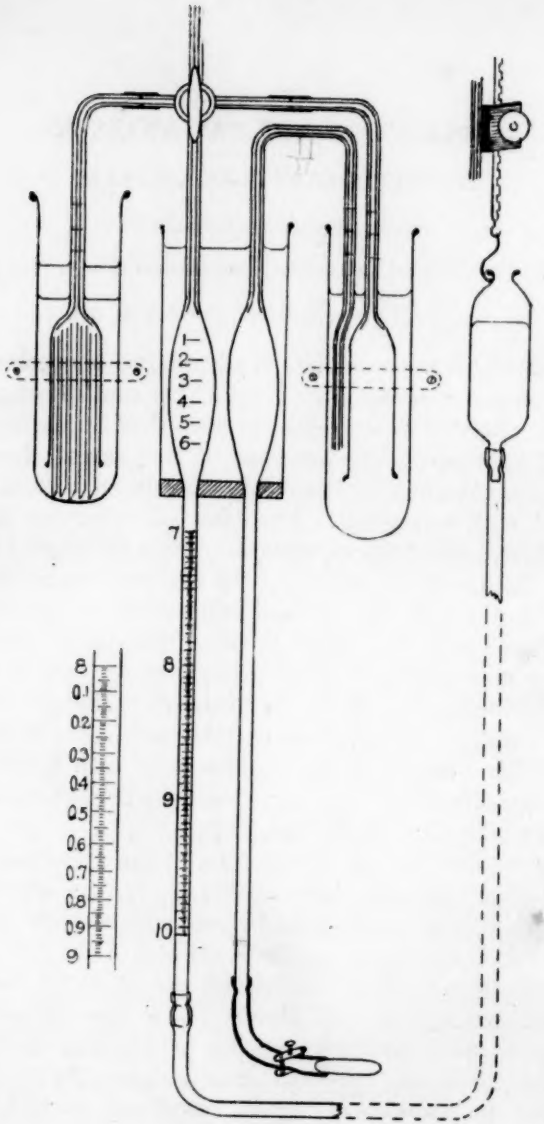

FIG. 1.

the fatiguing elevations of the mercury bulb. In use the pyrogallate solution is covered with a layer of petroleum oil sufficiently deep to protect it from the outside air. The lower ends of the pieces of glass tubing in the pyrogallate must be cut or broken diagonally. The control tube may be directly back of the gas burette instead of to one side as in the figure.

It will be noticed that the water jacket enclosing the gas burette and the control tube has been shortened so as to include little more than the

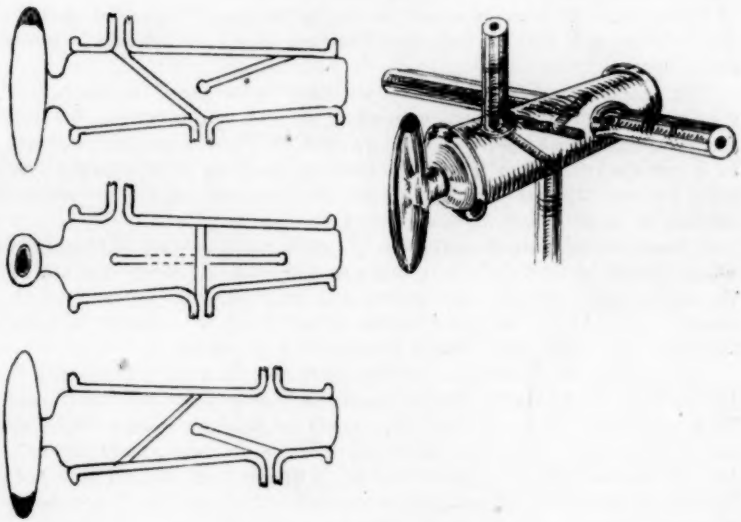

FIG. 2.

bulbs and upper parts. This is allowable because the object of the water jacket is not to keep the temperature constant but to make it possible by stirring or bubbling the water to bring the air in the two bulbs to the same temperature and water vapor tension at the time of an observation. It is extremely hard on the eyes to read a fine burette accurately through the glass and water of a long jacket. A small magnifying glass arranged to slide on the tube of the gas burette is convenient.[2] In all other respects the apparatus, the technique, and precautions on its use are the

[2] Wm. Gaertner and Company, 5345 Lake Park Avenue, Chicago, make a convenient form (Catalog No. C1365).

same as with the usual form of instrument.[3] Attention may however be called to one or two points which are often overlooked.

Red rubber should not be used on the connections where alkali may touch it, as it gives off sulfur which may finally appear as hydrogen sulfide in the burette.

To make the rubber connections tight (in case of leakage) it is convenient to wrap them with elastic bands. Loop the band around the rubber tube, pull tight, and wrap the free end around the tube several times. Pass a small curved forceps under the wrapping, catch the end of the rubber band over the points of the forceps, and twist the end under the wrapping by withdrawing the forceps. This is a much better method of making joints tight than wrapping them with wire.

The walls of the burette should always be moistened with a fraction of a drop of 1 per cent sulfuric acid, and 2 or 3 cc. of this fluid should be placed in the control tube.

The analyst must remember that accuracy in the use of the instrument does not depend upon an extreme effort to read the burette as finely as possible, but rather upon making sure that the absorptions are complete. It is essential therefore frequently after an analysis is supposedly complete to pass the gas over again into the absorbent and make another reading to be sure that no change occurs.

A check on calibration, tightness of joints, and efficiency of absorbents which should be used daily is to make an analysis of atmospheric air. If the apparatus is properly graduated and in good order the sum of the oxygen and CO_2 in uncontaminated atmospheric air should be found 20.96 per cent, with an allowable error of $\pm$ 0.03 per cent.

The method of graduation used by most American glass blowers is far too rough and inaccurate for the precision requisite for this instrument. Thus in some burettes of this type made in America I have found the errors of graduation ten or even twenty times as great as are allowable (giving an analysis of ordinary air at 21.20 per cent oxygen or worse). The absolute error of the instrument was only 0.1 cc. and this is not greater than is quite common and is allowable for common purposes in an ordinary titration burette. For the calibration of the gas burette the glass blower must not use water but mercury. The standard burette against which he calibrates must be of at least as fine bore as that of the tube of the burette to be calibrated. The standard burette must have been calibrated with weighed amounts of mercury and no mark on the burette purchased should have an error greater than 0.005 cc.

The purchaser of a burette should test it before assembling by inverting the burette, connecting it with a reservoir of mercury, filling to the 7, 8, 9, and 10 cc. marks, respectively, pouring this mercury into a small tarred beaker, and weighing. 1 cc. of mercury at room temperature (18°C.) weighs 13.55 gm. An allowance of 0.004 cc. may be made for the reversal of the convexity of the meniscus in the inverted burette.

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[3] Haldane, J. S., Methods of Air Analysis, London, 1912.

The correctness of the graduation of the tube of the burette is most easily checked by drawing in on top of the mercury about 0.5 cc. of water. By raising and lowering the mercury and reading the air-water and water-

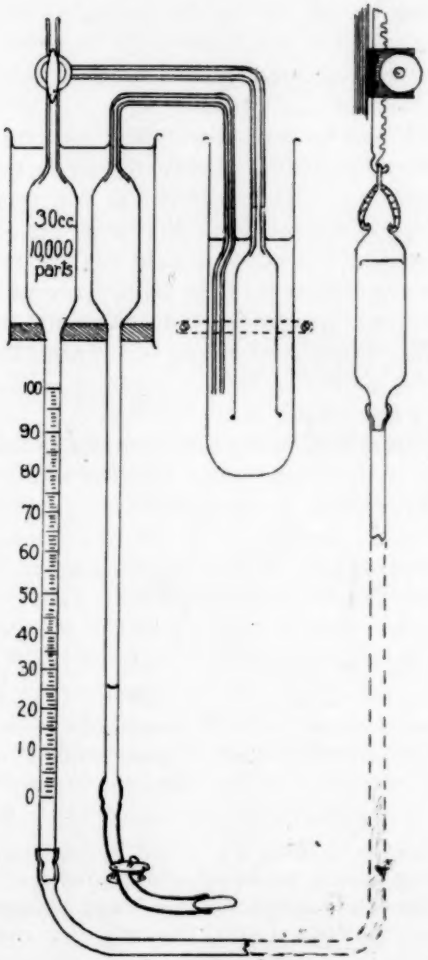


FIG. 3.

mercury menisci at various levels (allowing time for the water to drain from the sides), errors of graduation due to irregularities in the caliber of the tube are revealed. The difference in the volume between the two menisci should not vary more than 0.005 cc. at any two levels.

In Fig. 3 is shown a similar modification of the Haldane analyzer for the CO_2 content of atmospheric air. The bulb of the burette for this apparatus should have a capacity of 30 cc., and the tube of the burette should be graduated up to 100 parts (0.003 cc. each) of absorbable gas (CO_2) in 10,000 of air. This instrument is quite as accurate and much simpler and cheaper than the Pettersson-Palmquist analyzer.

In Fig. 4 is shown an apparatus which has been found useful for teaching purposes in this laboratory (one instrument to each six or eight students). For beginners it is a very much easier apparatus to use than the form shown in Fig. 1. Acidulated water (1 per cent sulfuric acid) is used in the burette instead of mercury. Common glass 6 ounce bottles are used to hold the absorbents, sodium hydroxide, and potassium pyrogallate. A layer of paraffin oil is placed on top of the absorbents to protect them from the air. The bottles stand on a shelf which also supports the water jacket of the burette.

The apparatus is used in the same manner as that described in the first paper of this series except that the addition of a control tube renders it possible to compensate for temperature changes in the course of an analysis. The use of the control tube is as follows. A strip of mm. paper is pasted on the leveling bottle. The top of the control tube is opened. The leveling bottle is lifted a few inches and the fluid allowed to rise part way up the control tube, the top of which is then closed. The leveling bottle is held so that the fluid is at the same level in the bottle and in the tube and a loop of wire (X) is placed at this height on the tube. Before each measurement of gas volume in the burette the leveling bottle is moved up or down the control tube, and by means of the mm. paper strip note is made of the height above or below the meniscus in the control tube at which the bottle must be held to bring the gas in the control tube to its original volume (at X). The leveling bottle is then held at the same height above or below the meniscus in the burette. In this way the gas in the burette can always be brought to the correct volume per molecule.

This instrument is adequate to all scientific and clinical investigations except those involving the most extreme accuracy. The elimination of mercury renders it very much easier to use.

If time is allowed for the fluid to drain from the walls of the burette before each reading oxygen analyses come out nearly as well as with the mercurial burette. With CO_2 the error due to

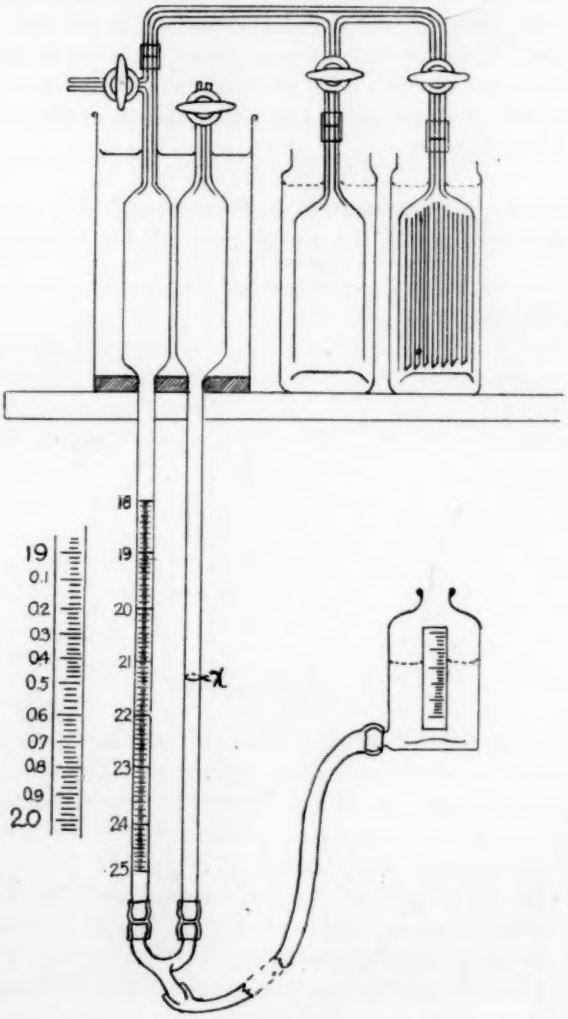


Fig. 4.

solution in the acidulated water is very close to 1 per cent; that is, analyses of air giving 5.50 per cent should be corrected to 5.55 per cent, or 2.10 to 2.12.

The following names may be used for the apparatus[4] described in this series of papers: (1) For that described in the first paper, analyzer for CO_2 in alveolar air and blood; (2) for that shown in Fig. 1 of this paper, respiratory gas analyzer; (3) for that in Fig. 3, atmospheric CO_2 analyzer; (4) for that shown in Fig. 4, modified Orsat analyzer.

I am indebted to Professor A. L. Prince and Mr. A. H. Smith for valuable assistance in the development of this apparatus.

[4] These instruments may be obtained from the Emil Greiner Company, 55 Fulton St., New York.

APPLICATIONS OF GAS ANALYSIS.

V. BLOOD GAS ANALYSIS.

By YANDELL HENDERSON and ARTHUR H. SMITH.

(*From the Physiological Laboratory, Yale Medical School, New Haven.*)

(Received for publication, October 30, 1917.)

The method to be described here is essentially a modification of that of Barcroft and Haldane (1) and of that described in the first paper of this series (2). The solutions employed are: (1) dilute ammonia (1 cc. of concentrated ammonia in 500 cc. of distilled water) kept tightly stoppered to avoid absorption of CO_2 from the air (or freed from carbonate with barium hydroxide and ammonium sulfate (3)). A little saponin is dissolved in the ammonia solution when an oxalate is used to prevent the blood sample from clotting. (2) A 10 per cent solution of potassium ferricyanide. (3) A 20 per cent solution of tartaric acid.

The apparatus used consists (1) of a Haldane gas analyzer in either its original or modified form (4), and (2) a small tube such as is shown in Fig. 1.

Oxygen Analysis.

The stop-cock of the diffusion tube is closed (*i.e.*, turned to connect the side and end nipples); 1.5 cc. of the ammonia solution are placed in the tube; and 1 cc. of the blood to be analyzed is introduced below the ammonia by means of a pipette. A short close fitting rubber stopper is inserted in the large end. A hypodermic needle (disconnected from its syringe) is passed through this stopper to allow the escape of the air compressed by the insertion of the stopper. The needle is withdrawn, and the tube is rotated gently to mix the blood and ammonia until the corpuscles are completely laked. A small all glass syringe fitted with a hypodermic needle is then filled with the ferricyanide solution and the air is expelled. The needle is then thrust through the

rubber stopper until the point projects in the interior of the tube, and 0.25 cc. of the ferricyanide solution are injected. The needle is then carefully withdrawn.

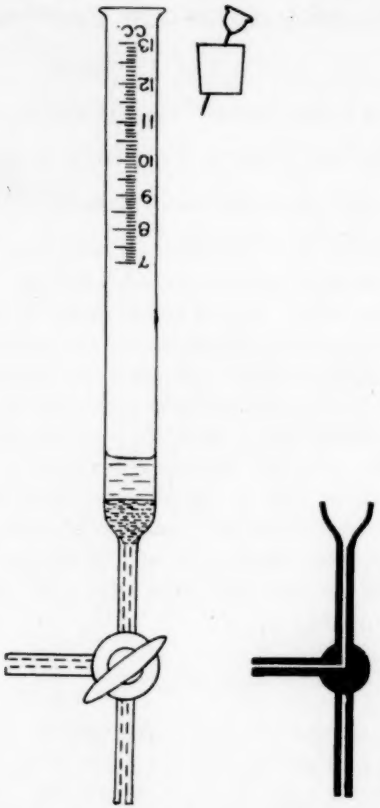


FIG. 1. The blood gas diffusion tube. The plug of the stop-cock is bored only in a right angle, not in a T, so that only two of the openings are connected at once. The open end of the tube is graduated as shown. This end of the tube is closed with a rubber stopper cut to about a half or a third the usual length, so that the needle of a small all glass hypodermic syringe can be thrust through it.

The tube is now placed for 5 minutes in a rotating apparatus (Fig. 2) consisting of a block of wood turning on an iron rod and belted to a slowly moving motor or shafting. There are a num-

ber of holes bored in the sides of the block of wood into which diffusion tubes can be inserted. The rate of revolution should be not more than 40 or 50 times a minute. During the rotation the contents of the tube spread in a thin film along the walls and allow complete diffusion of the liberated oxygen into the air of the tube.

The tube is next connected glass to glass with the gas analyzer by a short piece of heavy rubber tubing in the manner shown in Fig. 3. The mercury bulb of the analyzer is lifted until mercury runs out through the top of the analyzer and fills the capillary tube of the blood gas diffusion tube. The lower end of the diffusion tube is placed in a beaker of water and the stopper is

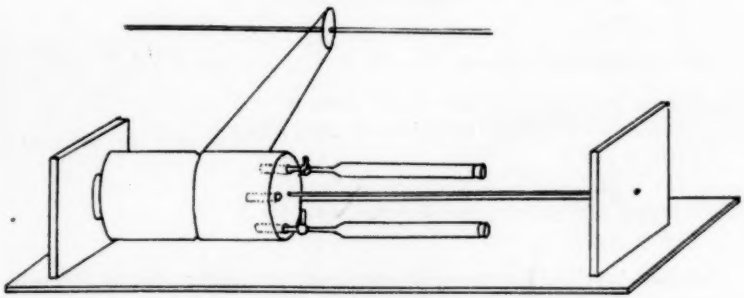


FIG. 2. Rotating apparatus.

withdrawn under water. The beaker is raised or lowered until the levels of the fluid inside and outside the tube are the same, and the volume of the air in the tube is read. The stop-cock at the top of the diffusion tube is then turned and a sample of the air within the tube is drawn over into the analyzer and analyzed for oxygen.

The residual gas from this analysis is nitrogen. As the percentages of oxygen and nitrogen in atmospheric air are known, it is easy to calculate from the volume of this residual nitrogen the exact amount of oxygen which would have been found by the analysis if no oxygen had been given off by the blood. To do this multiply the residual nitrogen by $\frac{20.93}{79.04}$, or 0.265. The volume of oxygen thus calculated to have been in the air is sub-

tracted from that found. A correction is then made for the volume of gas which remained in the diffusion tube and connections after the sample was drawn. Corrections are also made for barometric pressure and temperature, for the volume of gas is always expressed as it would be at 0°C. and 760 mm. barometer. The following is an example of this calculation.

Volume of air in diffusion tube	= 10.1 cc.	(1)
Volume taken for analysis	= 9.440 "	(2)
Nitrogen remaining after absorption of oxygen.	= 7.342 "	(3)
Oxygen absorbed, *i.e.*, (2)–(3)	= 2.098 "	(4)
Volume of atmospheric oxygen in volume of gas taken for analysis, *i.e.*, (3) $\times \frac{20.93}{79.04}$	= 1.945 "	(5)
Oxygen from blood in volume of gas analyzed, *i.e.* (4)–(5)	= 0.153 "	(6)
Total oxygen from blood, *i.e.*, (6) $\times \frac{(1)}{(2)}$	= 0.163 "	(7)
Oxygen reduced from barometric pressure 755 and temperature 20° to 760 and 0°, *i.e.*, (7) $\times \frac{760}{755} \times \frac{273}{(273+20)}$*	= 0.147 "	(8)
Volumes per cent oxygen in blood, *i.e.*, (8) $\times$ 100	= 14.7	

* Tables for these factors of pressure and temperature will be found in any handbook of chemical constants.

Comparative determinations of the oxygen content of the blood by the Barcroft-Haldane method using the Brodie apparatus (5) and by our method gave the following results.

Barcroft-Haldane method.	Gas analysis method.
16.2	16.3
16.5	16.1

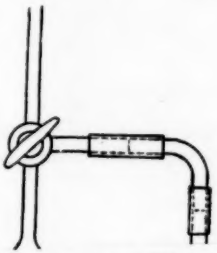
The two methods are we believe of approximately the same order of accuracy. To one accustomed to the use of the gas analyzer, but not in practice for the use of the Barcroft-Haldane method in its usual form, our modification is, we believe, decidedly the easier.

The precision of the method may be increased,—at least theoretically—by filling the diffusion tube initially with nitrogen (from the analyzer) instead of air, inserting the stopper under

water, and injecting the blood with the hypodermic syringe. But this refinement takes time and requires extreme care to avoid admitting the least trace of air.

CO_2 Determination.

The CO_2 content of the blood is determined in exactly the same manner and with the same tube, merely using the tartaric



CORRECTION.

On page 42, Vol. XXXIII, No. 1, January, 1918, the 18th line, for $\frac{760}{755}$ read $\frac{755}{760}$.

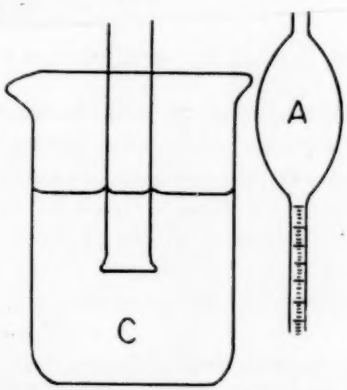


FIG. 3. A, gas burette of analyzer; B, diffusion tube; C, beaker of water.

acid solution instead of ferricyanide. (When the amount of gas in the blood is very high it is advisable to use only half quantities of blood and reagent; namely, 0.75 cc. of ammonia, 0.5 cc. of blood, and 0.10 cc. of tartaric acid.) Owing to the solubility of CO_2 it is necesary in the calculation of the results to take into account that part of the gas which remains in solution in the

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Volumes per cent oxygen in blood, *i.e.*, (8) $\times$ 100	= 14.7		

C

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Comparative determinations of the oxygen content of the blood by the Barcroft-Haldane method using the Brodie apparatus (5) and by our method gave the following results.

Barcroft-Haldane method.	Gas analysis method.
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The precision of the method may be increased,—at least theoretically—by filling the diffusion tube initially with nitrogen (from the analyzer) instead of air, inserting the stopper under

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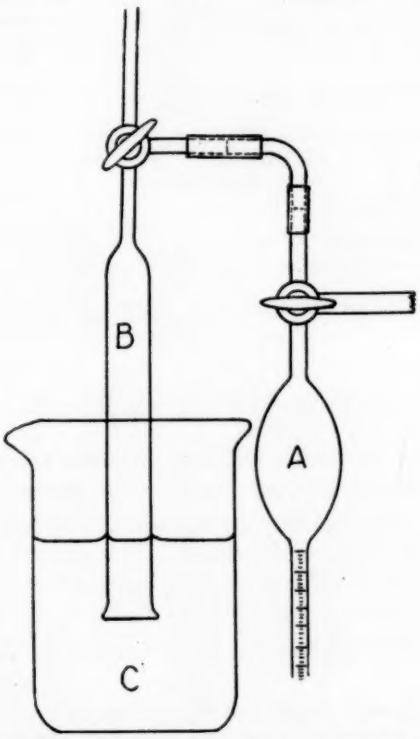


FIG. 3. A, gas burette of analyzer; B, diffusion tube; C, beaker of water.

acid solution instead of ferricyanide. (When the amount of gas in the blood is very high it is advisable to use only half quantities of blood and reagent; namely, 0.75 cc. of ammonia, 0.5 cc. of blood, and 0.10 cc. of tartaric acid.) Owing to the solubility of CO_2 it is necesary in the calculation of the results to take into account that part of the gas which remains in solution in the

acidified diluted blood. This correction for any temperature may be obtained from Curve 1 in Fig. 4.

A typical calculation of the results obtained is as follows.

Volume of air at 18°C. (compressed) in diffusion tube before removing stopper	= 10.9	cc.	(1)
Volume of air (at atmospheric pressure) in diffusion tube after removing stopper	= 11.1	"	(2)
Volume taken for analysis	= 7.774	"	(3)
Gas remaining after absorption of CO_2	= 7.506	"	(4)
CO_2 absorbed, *i.e.*, (3)–(4)	= 0.268	"	(5)
Total CO_2 in air in tube, *i.e.*, (5) $\times \frac{(2)}{(3)}$	= 0.383	"	(6)
CO_2 in solution in acidified blood, *i.e.*, volume of liquid (2.75 cc.) $\times$ solubility coefficient (1.0) $\times$ CO_2 in air in tube $\frac{(6)}{(1)}$	= 0.096	"	(7)
Total CO_2 from blood, *i.e.*, (6) + (7)	= 0.479	"	(8)
CO_2 reduced from barometric pressure 762 and temperature 26° to 760 and 0°, *i.e.*, (8) $\times \frac{760}{762} \times \frac{273}{(273+18)}$	= 0.448	"	(9)
Volumes per cent CO_2 in blood, *i.e.*, (9) $\times$ 100	= 44.8		

Comparison of the results obtained with the original Barcroft-Haldane method and with our modification shows in every case that the latter gives figures 2 to 3 volumes per cent higher than those obtained with the older method. This is due to the fact that the heavy precipitate produced by adding both ferricyanide and acid to blood renders it extremely difficult in the Brodie apparatus to shake the last trace of CO_2 out of the thick coagulum.

Simultaneous Determination of Oxygen and CO_2.

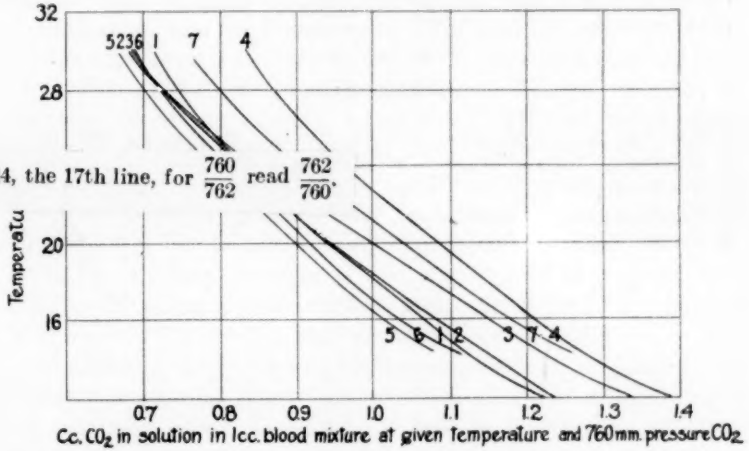
There appears no reason (except that just mentioned) why both gases should not be determined on a single sample of blood by first liberating the oxygen and then the CO_2. We have found that this can in fact be done (using half quantities of blood and reagents or in diffusion tubes of 20 cc. capacity) if a small glass rod or some lead shot is put into the tube to assist in breaking up the heavy masses of the precipitate formed by the addition

of the acid after the ferricyanide. Practically, however, unless the quantity of blood available is extremely limited it is easier and more accurate to make the two determinations separately. Duplicate determinations for both gases independently can be made by this method in half an hour.

Solubility of CO_2 in Acidified Blood Solutions.

As the correction for the amount of CO_2 which remains dissolved in the fluid is considerable we have devoted particular care

On page 44, the 17th line, for $\frac{760}{762}$ read $\frac{762}{760}$.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0.7 0.8 0.9 1.0 1.1 1.2 1.3 1.4
Cc. CO2 in solution in 1cc. blood mixture at given temperature and 760mm. pressure CO2

FIG. 4. Curves expressing solubility of CO_2.

The acid-blood mixture of Curves 1, 2, 3, 4, and 7 was made as follows: ammonia 1.5 cc., blood 1.0 cc., tartaric acid 0.25 cc. The mixture of Curves 5 and 6 was made as follows: ammonia 2.0 cc., blood 1.0 cc., tartaric acid 0.5 cc.

Curves 1 and 2, fresh human blood; Curve 3, fresh cat blood; Curve 4, blood of dog anesthetized with chloratone and alcohol; Curve 5, fresh pig blood; Curves 6 and 7, human serum.

to this matter. Various arrangements of apparatus were tried but finally one similar to that shown in Fig. 3 (except that the rubber tube connecting the diffusion tube and gas burette was 4 or 5 cm. long to allow shaking the tube) proved most convenient, as an entire series for all temperatures could be run from a single filling of the tube.

acidified diluted blood. This correction for any temperature may be obtained from Curve 1 in Fig. 4.

A typical calculation of the results obtained is as follows.

Volume of air at 18°C. (compressed) in diffusion tube before removing stopper	= 10.9 cc.	(1)
Volume of air (at atmospheric pressure) in diffusion tube after removing stopper	= 11.1 "	(2)
Volume taken for analysis	= 7.774 "	(3)
Gas remaining after absorption of CO_2	= 7.506 "	(4)
CO_2 absorbed, *i.e.*, (3)–(4)	= 0.268 "	(5)
Total CO_2 in air in tube, *i.e.*, (5) $\times \frac{(2)}{(3)}$	= 0.383 "	(6)
CO_2 in solution in acidified blood, *i.e.*, volume of liquid (2.75 cc.) $\times$ solubility coefficient (1.0) $\times$ CO_2 in air in tube $\frac{(6)}{(1)}$	= 0.096 "	(7)
Total CO_2 from blood, *i.e.*, (6) + (7)	= 0.479 "	(8)
CO_2 reduced from barometric pressu temperature 26° to 760 and 0°, *i.e.*, $\times \frac{762}{(273 + 18)} \times \frac{273}{}$	= 0.448 "	(9)
Volumes per cent CO_2 in blood, *i.e.*, (9) $\times$ 100	= 44.8	

Comparison of the results obtained with the original Barcroft-Haldane method and with our modification shows in every case that the latter gives figures 2 to 3 volumes per cent higher than those obtained with the older method. This is due to the fact that the heavy precipitate produced by adding both ferricyanide and acid to blood renders it extremely difficult in the Brodie apparatus to shake the last trace of CO_2 out of the thick coagulum.

Simultaneous Determination of Oxygen and CO_2.

There appears no reason (except that just mentioned) why both gases should not be determined on a single sample of blood by first liberating the oxygen and then the CO_2. We have found that this can in fact be done (using half quantities of blood and reagents or in diffusion tubes of 20 cc. capacity) if a small glass rod or some lead shot is put into the tube to assist in breaking up the heavy masses of the precipitate formed by the addition

of the acid after the ferricyanide. Practically, however, unless the quantity of blood available is extremely limited it is easier and more accurate to make the two determinations separately. Duplicate determinations for both gases independently can be made by this method in half an hour.

Solubility of CO_2 in Acidified Blood Solutions.

As the correction for the amount of CO_2 which remains dissolved in the fluid is considerable we have devoted particular care

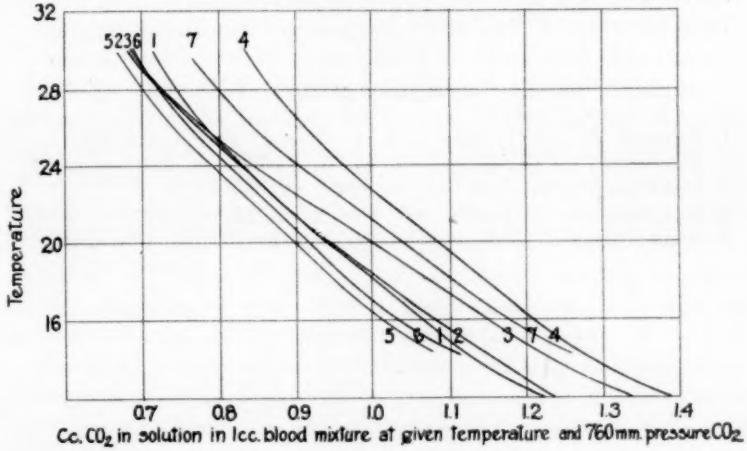
32
28
24
20
16
Temperature
5 2 3 6 1 7 4
5 6 1 2 3 7 4
0.7 0.8 0.9 1.0 1.1 1.2 1.3 1.4
Cc. CO2 in solution in 1cc. blood mixture at given temperature and 760 mm. pressure CO2

FIG. 4. Curves expressing solubility of CO_2.

The acid-blood mixture of Curves 1, 2, 3, 4, and 7 was made as follows: ammonia 1.5 cc., blood 1.0 cc., tartaric acid 0.25 cc. The mixture of Curves 5 and 6 was made as follows: ammonia 2.0 cc., blood 1.0 cc., tartaric acid 0.5 cc.

Curves 1 and 2, fresh human blood; Curve 3, fresh cat blood; Curve 4, blood of dog anesthetized with chloratone and alcohol; Curve 5, fresh pig blood; Curves 6 and 7, human serum.

to this matter. Various arrangements of apparatus were tried but finally one similar to that shown in Fig. 3 (except that the rubber tube connecting the diffusion tube and gas burette was 4 or 5 cm. long to allow shaking the tube) proved most convenient, as an entire series for all temperatures could be run from a single filling of the tube.

The method employed consisted in first filling the diffusion tube and burette with pure CO_2 gas from a Kipp generator. The rubber stopper was then inserted in the diffusion tube. The tube was immersed in water at various temperatures and the volume of gas read on the burette. 2 cc. of diluted acidified blood entirely freed from CO_2 were injected through the stopper into the diffusion tube. The tube was thoroughly shaken at various temperatures and the volume of gas going into solution was read from the burette. In the calculation allowance was made for the fact that the gas in the burette remained always at room temperature. The curves in Fig. 4 give the relative distribution of CO_2 at temperatures between 12° and 30°C. in gaseous form and in solution.

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APPLICATIONS OF GAS ANALYSIS.

VI. THE RESPIRATORY EXCHANGE AND INDIRECT CALORIMETRY.

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(Received for publication, October 30, 1917.)

The analysis of 10 cc. of expired air to determine its percentage of oxygen and CO_2 is not appreciably more difficult than the determination of the nitrogen in 10 cc. of urine by the Kjeldahl method. The nitrogen found in the sample multiplied by the total volume of the urine from which it was taken gives us the nitrogen metabolism of the body. In the same way the oxygen (deficit) and the CO_2 found in the sample of expired air multiplied by the total volume of the air expired in a given time afford the oxygen and carbon metabolism, and, by indirect calorimetry, the total energy exchange of the body.

Certainly a knowledge of the oxygen, carbon, and energy exchanges is quite as important in a wide range of problems as is the nitrogen exchange. Each should be equally a matter of routine in every well equipped biochemical and clinical laboratory. In fact however the means and habit of determining nitrogen are universal, while determinations of the respiratory exchange are confined to a very few highly specialized laboratories.

The reason for this anomaly lies in the fact that it is generally assumed that the elaborate and expensive apparatus of the "closed circuit" method used by Benedict, Lusk, and their collaborators is necessary. This is far from the truth. In fact, in a thorough comparative study and critique of all available methods Carpenter (1), although he does not say so explicitly, has shown implicitly that the (Tissot) "open circuit" method of collecting the expired air in a graduated spirometer and analyzing a sample is equal in accuracy and superior in simplicity to the closed circuit method.

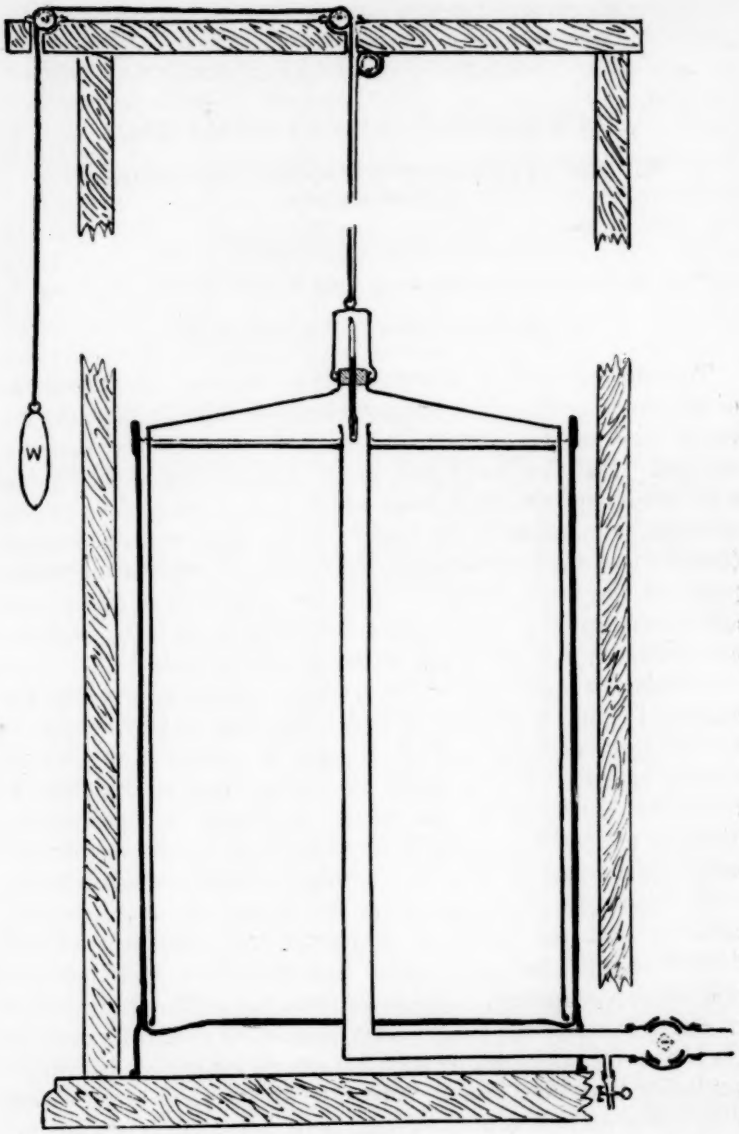

FIG. 1.

Apparatus for gas analysis has been discussed in the fourth paper of this series. In addition to a gas analyzer one needs a large graduated spirometer, a flexible tube, nose clip, and a mouthpiece with inspiratory and expiratory valves.

The spirometer shown in Fig. 1 is a simple form which has proved easily constructed and sufficiently accurate in use in this laboratory. The lower container consists of a common galvanized iron ash can. The tube is iron pipe (bore 1 inch) with a brass cock such as is used on steam-pipes. A round hole ($\frac{3}{4}$ inch) is bored in the side so that it serves as a 3-way cock. A disk of galvanized iron is fastened near the top of the pipe and lies just below the surface of the water with which the can is filled. This prevents the diffusion of any considerable amount of CO_2 into the water. The bell or floating container is made of tin. It should be as nearly as possible of exactly the same diameter everywhere (this requirement is easily met by any competent tinsmith). The cord to the counterpoise weight runs over window sash pulleys. The rise of the bell of the gasometer is measured by a self-winding steel mm. tape measure of which the case is fastened to the cross bar of the wooden frame. The volume of the bell per mm. rise is merely its diameter multiplied by πr^2. The error which would occur if the dead space of the apparatus were filled with fresh air is eliminated by running a fore period in each experiment during which the gasometer is partly filled with expired air. Just before the period of the observation it is emptied through the side hole in the brass cock, thus leaving the dead space of the apparatus (tube and top of bell) filled with expired air.

To connect the mouthpiece and the spirometer the best tubing is the corrugated rubber used in mine rescue apparatus, but this is expensive and a very satisfactory substitute is afforded by a piece of vacuum cleaner tubing, which can be ordered in the proper length (2 or 3 feet) with heavy rubber nipples at the ends, from any vacuum cleaner shop. The standard rubber and metal mouthpiece has mica disk inspiratory and expiratory valves attached to it.[1]

A form of valve which has proved convenient has recently been devised at the University of Minnesota by Professors A. D. Hirschfelder and E. D. Brown. By their kind permission a diagram of two of these valves arranged for inspiration and expiration on a T-tube is shown in Fig. 2. Each valve is made of a large tin

[1] These valves on a metal mouthpiece, the rubber mouthpiece and corrugated tube and nose clip, as well as the Douglas bag with a large aluminum 3-way cock and other similar equipment are obtainable from H. N. Elmer, 1140 Monadnock Building, Chicago, the agent for Siebe, Gorman & Company of London.

salve box with a disk of sheet rubber inside, held in place by a ring of spring wire soldered to the box at one point. The crack round the box is made tight with adhesive plaster. In the figure the cover of one valve is removed to show the rubber and wire.

A double valve (inspiratory and expiratory) which also has been used in this laboratory is shown in Fig. 3. It is made of two pieces of brass tubing ($1\frac{1}{2}$ and 1 inch interior diameter respectively) arranged concentrically. The moving part of each valve is a strip of heavy sheet rubber inside the tube covering holes

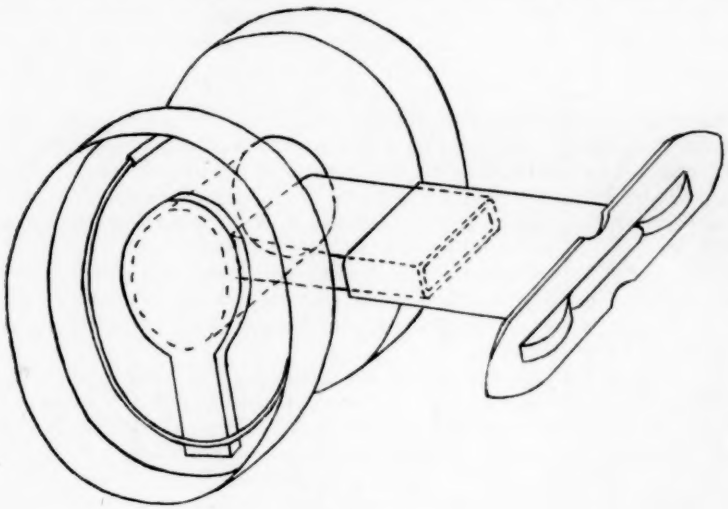

FIG. 2.

bored through the tubing and held in place by a metal clip near the middle of the strip. The lower ends of the tubes are closed with paraffined corks.

Perhaps no better idea can be given of the extensive use which can be made of the open circuit method combined with gas analysis than by reproducing here the directions given to the students in the routine course of physiology in this laboratory. These directions are typewritten together with the tables mentioned and are pasted upon pieces of compo board so that they can be conveniently carried about to the points where work is being done.

For the experiments in which the subject remains in one place the spirometer is used; for those in which he walks about the Douglas bag (2) is employed. The three tables referred to are as follows.

Table I.—For reduction to dry air at 0° and 760 mm. of mercury of 100 volumes of air saturated with moisture at different temperatures and pressures. This is reproduced from page 55 of Haldane's *Methods of Air Analysis* (3).

Table II.—For transforming apparent into true respiratory quotients. This is reproduced from page 57 of Haldane's *Methods of Air Analysis.* (The table includes one error; in the column of true respiratory quotients, 1.031 should be 1.131.)

Table III.—For the heat value of 1 liter of oxygen consumed in the body at various respiratory quotients. This is taken from page 357 of Volume xii of this *Journal* (4).

The directions are as follows.

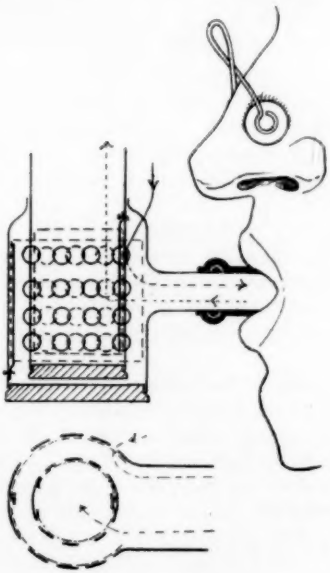

FIG. 3.

Calculation of Results of Experiments on Respiratory Exchange with Spirometer, Douglas Bag, and Small Animal Respiration Apparatus.

Analyze sample of air from the spirometer or bag for percentage of oxygen and CO_2. The volume of the gasometer is obtained by knowing the diameter of the bell, calculating its cross-section in sq. cm. by πr^2, and reading the distance the bell has risen on the self-winding steel tape measure fastened at the top of the bell. If the bag is used, measure contents of bag by passing the air through a gas meter and note temperature in the meter. Read barometer. By means of Table I reduce the volume of air expired in 1 minute to standard pressure and temperature (0°C. and 760 mm.).

From the CO_2 percentage subtract the CO_2 of the inspired air (0.03), and multiply by the (reduced) volume of air expired in 1 minute. This gives the CO_2 production per minute.

"The volume of oxygen absorbed is less easy to calculate however, as the volume of dry air has diminished in the process of respiration because more oxygen has been taken up than carbon dioxide has been given off. Since nitrogen is neither taken up nor given off in respiration, it is evident that for every 100 volumes of expired air there corresponded in the inspired air, not 20.93 volumes of oxygen, but $\frac{20.93}{79.04}$, or 0.2648, multiplied by the percentage of nitrogen in the expired air. By subtracting from the oxygen figure so obtained the oxygen percentage found by analysis, the true per cent of oxygen consumed is found. Multiplying the volume of air expired per minute by this percentage gives the oxygen really consumed, and dividing the CO_2 produced by the oxygen consumed gives the respiratory quotient" (Haldane, 3).

Instead of making this calculation, the results may be obtained by means of Table II. By this table the apparent respiratory quotients [CO_2 per cent found by analysis divided by (20.93 minus oxygen found by analysis)] can be transformed into true quotients, and the correct intake of oxygen obtained by dividing the output of carbon dioxide by the true respiratory quotient.

From Table III for indirect calorimetry the amount of energy produced and the character and amounts of the substances oxidized in the body are obtained by taking the figure corresponding to the respiratory quotient and multiplying it by the oxygen consumption.

To calculate the respiratory dead space (5) get the tidal volume (*i.e.*, total expired air divided by number of breaths) and multiply by

$$1.00 - \frac{\text{CO}_2 \text{ per cent in mixed expired air}}{\text{CO}_2 \text{ per cent in alveolar air}}.$$

The dead space for oxygen is obtained similarly. The oxygen pulse (6) is the oxygen consumed per minute divided by the pulse rate.

The external work on the stationary bicycle (7) equals the product of the resistance in kilos by the circumference of the wheel in meters by the number of revolutions per minute.

The object of this paper (the last of the series) has been to make more easily available facts and methods already known. The object of the entire series has been to emphasize the wide range of methods and problems for which gas analysis is a master key.

The expenses of all the investigations of this series of papers have been defrayed by the Loomis Medical Research Fund.

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A STUDY OF THE DIETARY ESSENTIAL, WATER-SOLUBLE B, IN RELATION TO ITS SOLUBILITY AND STABILITY TOWARDS REAGENTS.

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(From the Laboratory of Agricultural Chemistry of the University of Wisconsin, Madison.)

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Since the discovery by Eijkman (1) of a means of producing an experimental polyneuritis in birds by feeding them a diet restricted to polished rice, and its relief by feeding rice polishings, interest in the problem of the isolation and identification of the curative substance has steadily increased (2). Without exception the methods which have been adopted for the isolation of the physiologically active substance have involved extracting it with either water or alcohol and subsequent precipitation with phosphotungstic acid, mercuric chloride, or silver nitrate and barium hydroxide. The former reagent has been employed extensively. The decomposition of the phosphotungstic precipitate by means of alkali such as barium hydroxide has been found to be attended by great loss of curative properties of the preparations. Better success has attended the use of barium acetate and of lead acetate for this decomposition (3).

The various modifications of the methods of separation, which were based upon precipitation with reagents, have yielded but meager results, and it seemed to us that prospects for success in the isolation of this interesting substance must be based upon an entirely different line of procedure from that employed in the past. It was with a view to the development of a new method for the separation of the physiologically indispensable substance which we term water-soluble B (4), and which we believe to be the specific complex, a lack of which causes the development of polyneuritis, that the experiments reported in this paper were conducted. The plan contemplates a complete study of the solubility of the water-soluble B in all organic solvents available.

We sought, as suitable material with which to work, a vegetable product which, after the removal of fats by ether, gave promise of yielding the smallest amount of alcohol-soluble matter. The navy bean was selected, but this seed we now feel will not prove superior to a number of others which might have been employed. The bean is extremely difficult to grind to a fine state and after ether has removed everything which it is capable of extracting, alcohol still extracts about 4 per cent of the original weight of the bean. The bean is, however, very rich in the dietary B (5), and, as will be shown later, there seems good reason to expect that a method can be developed for separating the desired substance in a greatly concentrated form.

The navy beans were ground and dried, then exhausted with ether. This solvent does not take up a demonstrable amount of the water-soluble B. The ether-extracted material was subsequently extracted as described in the experimental part of this paper. Wheat embryo similarly treated was employed in some of the experiments.

We believe our method which consists of testing preparations for the presence of the antineuritic substance, water-soluble B, by relieving the polyneuritic condition and inducing subsequent growth in a mammal, to be greatly superior to the conventional method of "curing" pigeons which have been fed an exclusive diet of polished rice until they develop the disease. Polished rice is very deficient from the dietary standpoint in certain mineral salts, the fat-soluble A, and protein in addition to the specific substance water-soluble B, the lack of which leads to the development of polyneuritis (6). Recovery cannot be permanent on such a diet, and the observation of the "curative" effects of the administered substances is necessarily limited to a brief period.

We decided to adopt the rat instead of the pigeon as an experimental animal, because of the fact that the element of growth can be included in the observations. Where growth takes place and is sustained, there can be no possible doubt that we are supplying a chemical complex which is necessary as a *food* rather than a complex which produces its effects through pharmacological action. In the work with pigeons, previously reported (4), we had ample experience to convince us of the prompt recovery of birds from the polyneuritic state when given various

preparations. Careful reading of the literature relating to studies on polyneuritis in birds revealed so many confusing statements that we decided to attempt to perfect the technique for employing the rat for such studies. We feel convinced that a satisfactory procedure has been developed.

Funk (7) says that when the curative fraction obtained from either dry yeast or rice polishings was administered orally or subcutaneously to beri-beri pigeons, "the animals recovered very speedily, often in 2–3 hrs., but it was found impossible to keep them permanently on polished rice even when injections were repeated every few days."

Williams (8) has pointed out, and our experience harmonizes with his, that pigeons restricted to a diet of polished rice do not all run the same course. Many never develop acute polyneuritis, but waste away and die of starvation. Such birds are of no value in experimental work. Some, he states, recover temporarily from an acute attack without any treatment.

Eijkman (9) injected doses of 20 to 40 mg. of a mixture of one part NaCl and three parts KCl into chickens and pigeons and observed cures in pigeons but not in chickens. These results seem to justify the belief that temporary relief from paresis is less satisfactory as a test for the physiologically indispensable substance water-soluble B than is our method in which the diet is properly planned so as to be satisfactory for growth except for the absence of the one unidentified substance. With our diet resumption of growth actually takes place on the addition of the water-soluble B to the diet.

Antineuritic properties have been attributed to nicotinic acid (10) and to adenine (11), betaine, allantoin, and certain pyrimidines (12), and Williams has observed curative action in a certain isomer of α-hydroxypyridine (8). He has attempted to account for all of these observations by assuming that the needs of the animal are for a specific type of labile isomerism rather than a specific chemical complex as in the case of certain amino-acids in their relation to protein metabolism. That this explanation should be the correct one we believe improbable. The antineuritic substance employed by Williams was relatively labile, and passed easily into a form which possessed no curative properties. In the natural foods the evidence available from well

controlled experiments all indicates that the dietary essential termed by us the water-soluble B is not readily destroyed by aging, exposure to the atmosphere, or by heat at 100–112°C. (5) in neutral reaction. The experience of all investigators who have employed precipitation methods for the concentration of this substance indicates that it is very stable in dilute acid solutions. Indeed the statement has been made by Vedder and Williams (13) and by Funk that hydrolyzed preparations were more active than before hydrolysis.

It is possible to suggest an alternative explanation for Williams' hypothesis which will account for the occasional relief of polyneuritic pigeons by such a list of unrelated substances as nicotinic acid, certain pyrimidines, certain purines, and certain hydroxypyridines. The following explanation, while purely speculative, may possibly account for the recorded observations and assist in clarifying the confusing data relating to this subject.

Histological methods have shown that in polyneuritic animals there is a degeneration of the motor cells of the cord. This change is progressive; some cells present the normal appearance while others in the same field are degenerated. It would appear plausible that when the motor cell changes have reached a certain point loss of function supervenes and paralysis results. There still remain in the cord of a paralyzed animal motor cells which appear normal when stained and which may be capable of restoring the motor functions of the muscles when influenced by substances which stimulate them to heightened sensitiveness. In other words, the temporary relief of polyneuritis may be the result of the pharmacological action of certain substances rather than a response with renewed function of cells which have been subjected to a selective fast and later have been supplied with the missing food complex. If this line of reasoning could be shown to be valid, it would follow that experiments with pure chemical substances of known constitution, with a view to finding by good fortune the one playing an important physiological rôle, might be entirely misleading *unless it were shown that the "cure" was permanent.* For such complete proof it is necessary to demonstrate the resumption of growth and maintenance of health as long as the substance is supplied in the food mixture. Animals which have been brought into a critical condition where death is

certain within 24 hours, and which have been permanently cured on the addition to the food mixture of a very small amount of a preparation obtained in efforts to isolate the unidentified food factor, furnish absolute proof that the physiologically active *dietary factor* is being dealt with. Sustained normal function is indispensable to adequate proof that the dietary essential in question is being administered. If this condition should be insisted upon by investigators before concluding that a test is positive, it is probable that we should not have such a list of totally unrelated chemical substances reported as protective against polyneuritis.

Method of Experimentation.

When young rats are placed upon a diet which is satisfactory in every respect except for the absence of either of the dietary factors, fat-soluble A or water-soluble B, a considerable gain in weight may take place during the first 3 weeks. This does not always happen, but with vigorous rats it is of frequent occurrence. After the 2nd or 3rd week, on the experimental diet, there is no further increase in weight and either a brief period of maintenance is followed by rapid decline or steady loss of weight begins and death follows after a variable period, unless the missing dietary essential is supplied. Loss of hair and feebleness are seen with either type of deficient diet.

When the diet is lacking in the water-soluble B, but is properly constituted in other respects, typical polyneuritis results in many of the experimental animals. Funk (7) holds the view that beri-beri has not been produced in animals other than man and birds, and he believes that only those species whose end-product of purine metabolism is uric acid can develop the disease. The rat in common with other mammals with the exception of man, the higher apes, and the curious exception of the Dalmatian dog recently observed by Benedict (14), excretes allantoin, and according to Funk's classification should not suffer from this disease. In our experience rats restricted to a diet which is in every way adequate except for the absence of the antineuritic substance, water-soluble B, suffer decline in weight and loss of muscular control, especially in the hind limbs, and die within a few hours unless the necessary complex is supplied. Histological

studies have shown that there is degeneration of the motor cells of the cord in animals restricted to such a diet. We see, therefore, reason to believe that our animals in the experiments described are actually in the polyneuritic state. When kept upon a diet of polished rice, young rats do not live long enough to become paralyzed owing to the deficiency of *polished* rice in respect to protein, inorganic elements, and both the A and B (6). Rats suffering from polyneuritis are promptly relieved by the administration of the water-soluble unknown. When young rats are fed the diet of purified foodstuffs, plus butter fat, as employed in this work, polyneuritis occurs ordinarily between the 30th to the 40th day.

Animals given a diet which is properly constituted except that it is lacking in the fat-soluble A, may increase in weight during the first 2 weeks, then they gradually lose weight, become emaciated, and suffer from edema of the eyelids (15). We have recently (16) described this condition as xerophthalmia and believe it is due to this specific dietary deficiency. There is no loss of motor function in these animals. Their eyes are very much irritated and they scratch them and thereby cause mechanical injury. Recovery is prompt when the missing dietary factor is supplied, otherwise permanent blindness ensues and death supervenes, usually within 3 months.

Since we have no chemical methods for the detection of either of these two unidentified dietary essentials we must employ biological tests as a guide to their isolation. The period required in order to make the test complete must extend over several weeks because of the ability of the more vigorous animals to grow to a slight extent during the first 2 or 3 weeks, even when the substance to be tested for is absent from the diet. Preparations made with a view to separating the factor B in a pure form frequently led, if fed from the beginning of the experiment, to long maintenance and confusing data. This is due, on the one hand, to the difficulty, if not impossibility, of making complete extractions, so that the residues still contain traces of the factor B, and, on the other hand, to the fact that in all probability solvents in which this substance is regarded as insoluble remove small amounts when the extractions are greatly prolonged. Such residual amounts may well be wholly inadequate to cause recovery of

an animal in a serious pathological state, but, when furnished from the beginning of the feeding experiment, may extend the period of growth or maintenance and delay the appearance of signs of failure.

In conducting the work reported in this paper, we followed the plan of feeding a diet of purified food substances together with 5 per cent of butter fat to supply an abundance of the fat-soluble A. This diet was complete except that it was free from the water-soluble B. The rats were confined to this food mixture until they either had become stationary in weight or were declining. By the 5th week nearly all were either stationary in weight or were failing and they almost invariably showed signs of paralysis at about this time. When the rats were thus prepared, the material to be tested for the water-soluble B was put into the diet. The animals then either continued to decline or responded with growth. This method served to show within 2 weeks whether the substance B in significant amount was in the preparation under investigation. This procedure makes the test decisive. It also greatly shortens the time required to make the test.

The scheme of applying successively to *raw* navy beans the solvents ether, benzene, and 95 per cent alcohol, in the order named, and of feeding in separate experiments the extract and residue in the case of benzene and alcohol showed that benzene does not remove the water-soluble B from ether-extracted beans while hot alcohol does. The extraction with hot alcohol is not complete in a Soxhlet apparatus in 18 hours.

A surprising result was observed when the material which was dissolved from beans by hot alcohol was deposited on dextrin and the latter then extracted with hot benzene. The *benzene-soluble* material obtained by this procedure was very effective in inducing growth and in causing the prompt recovery of rats in the polyneuritic state. A benzene extract prepared directly from ether-extracted beans, however, does not contain an appreciable amount of the substance B. Benzene, therefore, does not remove the water-soluble B from beans directly, but once this substance is extracted by alcohol it is soluble in benzene.

Acetone does not extract the water-soluble B when the solvent is applied directly to the ether-extracted beans, and extracts only traces when applied to the alcohol-soluble matter from beans.

It is, therefore, possible to exhaust beans successively with ether, benzene, and acetone, and then to extract the physiologically active substance with alcohol. If the alcohol is then evaporated upon some substance, as dextrin, to distribute the alcohol-soluble material over a large surface, the desired substance can then be extracted with benzene (17). It is obvious that by this procedure and with the introduction of still other solvents the substances which accompany the physiologically active compound can in great measure be eliminated. By the introduction of precipitation methods at that point where interfering substances have been eliminated as far as possible, the chances of success in the preparation of a pure product or the preparation of derivatives, and with a minimum of loss in manipulation, are much greater, we believe, than in the methods of procedure hitherto employed by others.

In the experimental part of this paper it is shown that the water-soluble B is not extracted directly from beans, wheat germ, or pig kidney by ether, benzene, or acetone, but is readily extracted in great part by alcohol. After being removed by alcohol it is shown to be soluble in benzene, but very slightly soluble in acetone. The probability that there should be two or more physiologically indispensable substances in what we term water-soluble B, both or all of which should show the same solubility relations with three solvents, is relatively small and lends support to our view that the substance which protects animals against polyneuritis is the only essential complex in the extracts described. In other words, the data support the view that there are no specific substances present in these extracts which protect against such diseases as scurvy, rickets, pellagra, sprue, etc., and tend to confirm our contention that the latter are not due to specific starvation as is the case with beri-beri and xerophthalmia (16).

In interpreting the charts it should be remembered that at the point marking the beginning of the second period the animals were at or near the point of showing loss of muscular control, and that response with even a slow rate of growth on the addition of a preparation is conclusive evidence of the presence of the physiologically active substance, water-soluble B.[1]

[1] Credit is due to Mr. H. Steenbock for the preparation of the extracts employed in this work.

In the following charts, the diet in *Period 1* consisted of purified foodstuffs plus 5 per cent of butter fat.

Casein	18.0
Salt mixture 185	3.7
Agar-agar	2.0
Dextrin	71.3
Butter fat	5.0

Composition of Salt Mixture 185.

	gm.
NaCl	0.173
$MgSO_4$ (anhydrous)	0.266
$Na\ H_2PO_4.H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2.H_2O$	0.540
Fe citrate	0.118
Ca lactate	1.300

Chart 1.[2] *Lots 953 A, 952 A, and 952 B.*

Lot 953 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus the benzene extract of ether-extracted *raw* navy beans equivalent to 30 gm. of beans per 100 gm. of ration.

Period 3.—Ration: Same as Period 1 plus the benzene extract of the alcohol-soluble part of ether-extracted *raw* beans, equivalent to 30 gm. of beans per 100 gm. of ration; *i.e.*, alcohol-soluble,[3] benzene-soluble part of the bean.

This experiment shows that the water-soluble B is extracted from *raw* beans by hot alcohol, but not by ether or warm benzene. After the substance has been extracted from beans by means of alcohol, it is, however, soluble in benzene (see Chart 2, Lot 953 B, Period 4).

[2] The curves presented in this and the following charts are actual records of animals, and are representative of a group usually of four individuals. For economy of space a single record is shown. The broken curves marked N represent the normal expectation of growth.

[3] Only 95 per cent alcohol was employed in making the alcoholic extracts used in these experiments.

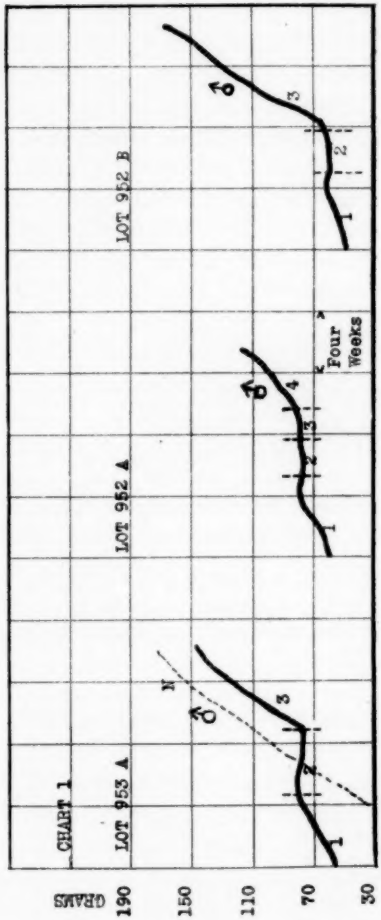


CHART 1
LOT 953 A
LOT 952 A
LOT 952 B
GRAMS
190
150
110
70
30
Four Weeks

Lot 952 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus the alcohol-soluble portion of ether-extracted *raw* navy beans evaporated on dextrin and extracted with acetone; *i.e.*, alcohol-soluble, acetone-insoluble part of beans. The ration contained the equivalent of 30 per cent of beans.

Period 3.—Ration: Same as Period 2 but with the alcohol-soluble, acetone-insoluble part of the *raw* beans heated 40 minutes at 15 pounds' pressure in an autoclave.

Period 4.—Ration: Same as Period 1 plus the acetone extract of the alcoholic extract of ether-extracted *raw* beans, plus the acetone-insoluble residue, *i.e.*, the equivalent of the total alcoholic extract, after being heated with acetone. The ration contained the equivalent of 30 per cent of beans (see preparation 951 B, under Chart 3).

In Chart 4, Lot 951 B, it is shown that growth could not be secured with the acetone-soluble part of the alcoholic extract of 30 per cent of *raw* beans in the diet. Since 10 per cent of beans supplies enough of the water-soluble B to induce growth (Chart 10, Lot 933) and neither the acetone extract or residue from the alcoholic extract of 30 per cent of beans in Lot 952 A, Chart 1 and Lot 951 B, Chart 4, induced growth, it seems certain that there is a slow destruction of the physiologically active substance in hot acetone.

Lot 952 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus 25 per cent of *raw* beans extracted with ether, then with benzene.

Period 3.—Ration: Same as Period 2, but with the beans moistened with distilled water and heated at 15 pounds' pressure for 40 minutes in an autoclave.

Neither ether nor benzene removes the water-soluble B from beans. In the second period the rats apparently did not grow because of failure to eat the ration containing raw beans. After heating the bean residue with water and thus making it more palatable, growth at once took place, which shows that the factor B was still in the residue.

Preparation of Extract and Residue Used with Lots 953 A and 952 B.—1,000 gm. of finely ground raw navy beans were extracted continuously for 25 hours with alcohol-free ether. The residue was then extracted with benzene in three successive 6

hour periods using fresh solvent each time to reduce the amount of exposure to heat. 1.635 gm. of material were extracted in the first two 6 hour periods and 0.222 gm. was extracted in the last. They were made up to 100 gm. with dried dextrin. The residue was freed from benzene and later heated in the autoclave. The benzene-soluble part was employed in the ration of Lot 953 A, Period 2. The benzene-insoluble part was used with Lot 952 B, Period 2, and, after heating, in Period 3. For the alcohol-soluble, acetone-insoluble fraction, employed in Chart 1, Lot 952 A, Period 2, see under 951 A, Chart 3.

Preparation of the Extract and Residue Employed with Lots 953 A, Period 3, and 953 B, Period 2.—1,000 gm. of finely ground *raw* navy beans were extracted for 25 hours with alcohol-free ether. 13.33 gm. of material were thus removed. The extracted residue freed from ether was then extracted with 95 per cent alcohol in three successive 6 hour periods. After distilling off the alcohol 30.45 gm. of extractives were left from the first two 6 hour periods and 9.7 gm. from the last. They were united in alcohol-water solution and evaporated on dextrin. Final weight of dry material, 165.1 gm.

The dextrin which carried the alcoholic extracts was transferred to a small Soxhlet apparatus and extracted with benzene successively in a 6, $6\frac{3}{4}$, and an 8 hour period. The extract from the first two periods weighed 25.39 gm., from the last period 0.61 gm. They were united, dried at 37°C., and ground up without evaporating on dextrin. This was fed to Lot 953 A, Period 3, Chart 1, and to Lot 953 B, Period 4, Chart 2.

The residue from the benzene extraction (alcohol-soluble, benzene-insoluble material) was made up to 200 gm. with dextrin. This preparation was fed to Lot 953 B, Period 2, Chart 2. It was later moistened with distilled water and heated 40 minutes in an autoclave at 15 pounds' pressure.

Chart 2. Lots 953 B, 994 A, and 994 B.

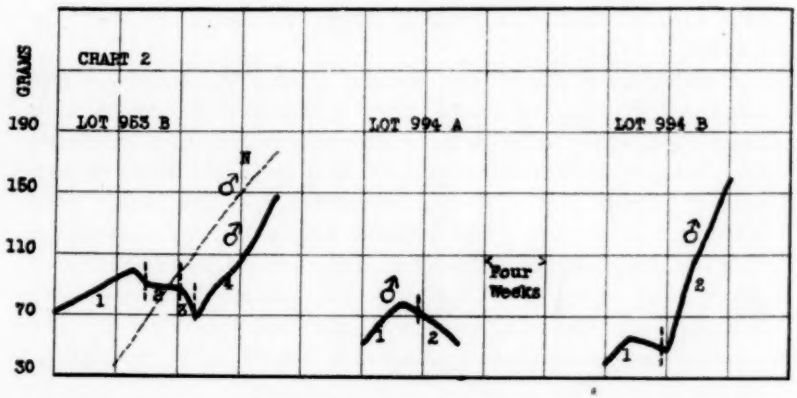
Lot 953 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus the portion of *raw* beans, soluble in alcohol but insoluble in benzene, equivalent to 30 per cent of beans.

Period 3.—Ration: Same as Period 2 but with alcohol-soluble, benzene-insoluble residue of *raw* beans heated 40 minutes in an autoclave at 15 pounds' pressure.

Period 4.—Ration: Same as Period 3 but with the benzene-soluble portion of the alcohol-soluble part of the beans included; *i.e.*, the total alcoholic extract of beans after treatment with benzene (used extract equivalent to 30 per cent of beans).

The alcoholic extract of *raw* navy beans contains the dietary essential water-soluble B. Although benzene does not extract this substance from the beans directly (Chart 1, Lot 953 A, Period 2; Chart 2, Lot 994 B), once it has been removed from the beans by means of hot alcohol, and the alcohol evaporated,



benzene dissolves the physiologically active substance fairly readily (Chart 1, Lot 953 A, Period 3; Chart 2, Lot 953 B, Period 4).

Lot 994 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 but with the benzene extract of ether-extracted *cooked* navy beans, equivalent to 30 per cent of beans in the diet.

Benzene does not take out the water-soluble B from the beans directly (Chart 1, Lot 953 A, Period 2). It must be first removed by hot alcohol, after which it is fairly soluble in hot benzene (compare Chart 1, Lot 953 A, Periods 2 and 3, and Chart 2, Lot 953 B, Period 4). Lot 994 B, Period 2, shows that the

factor B is still in the residue of the *cooked* beans after extraction with ether and then with benzene.

Lot 994 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 but with 25 per cent of navy beans (*cooked*) extracted with both ether and benzene.

This experiment shows that in Chart 2, Lot 994 A, Period 2, benzene failed to take out the water-soluble B from *cooked* beans, although the substance was still present after the thorough cooking. Lot 994 B shows that the residue of beans after extraction with ether and then with benzene still contains the unknown B. As we pointed out under Chart 1, Lot 953 A, Period 3, the substance is soluble in benzene after first being removed by alcohol.

Preparation of Extract and Residue Used with Lots 994 A and 994 B.—1,161 gm. of *cooked* navy beans were extracted with alcohol-free ether for 18 hours. The residue was then extracted with benzene in three consecutive 6 hour periods. The first two 6 hour periods gave 5.36 gm. of extract, the last period gave 0.904 gm. of extract. The extracts were taken up in benzene, in which they readily redissolved, and then were evaporated on dextrin and made up to 77.4 gm. The extract (benzene-soluble material) was fed in the ration of Lot 994 A, Period 2. The residue (benzene-insoluble) was fed in the ration of Lot 994 B, Period 2.

Chart 3. Lots 954 A, 954 B, and 951 A.

Lot 954 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus 25 per cent of the residue of *raw* navy beans after extraction with ether and then with acetone.

Period 3.—Ration: Same as Period 2 but with the bean residue moistened with distilled water and heated 40 minutes in an autoclave at 15 pounds' pressure.

Neither ether nor acetone removes the water-soluble B from raw beans (Chart 3, Lot 954 B, Period 2). There was no growth in Period 2 of Lot 954 A in which ether- and acetone-extracted *raw* beans were added to the diet, but this we attribute to failure of food consumption because in Period 3, after cooking the bean residue, which made it more palatable, rapid growth took place.

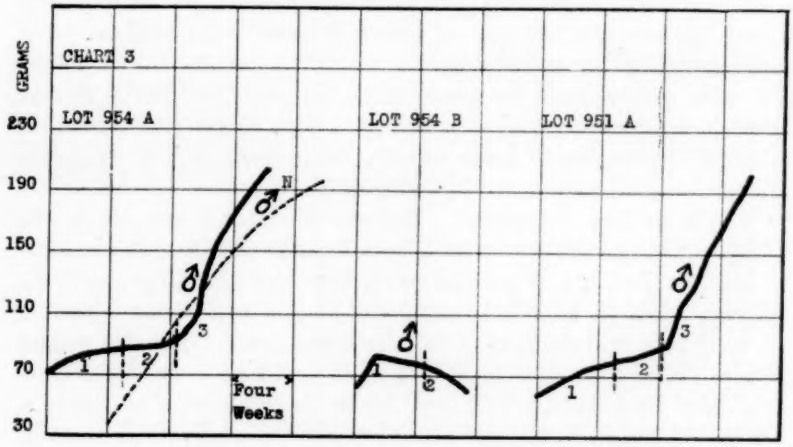
Lot 954 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 with an acetone extract of ether-extracted *raw* navy beans equivalent to 30 per cent of beans in the diet.

Acetone does not readily extract the water-soluble B from *raw* beans (see Chart 4, Lot 1,001 B, Period 2). The acetone-soluble part of the bean does not induce appreciable growth, but the acetone-insoluble *cooked* residue does (Chart 3, Lot 954 A, Period 3).

Lot 951 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 but with 25 per cent of the residue of *raw* navy beans after extraction with ether, then with alcohol.



Period 3.—Ration: Same as Period 2, but with the bean residue moistened with distilled water and heated in an autoclave 40 minutes at 15 pounds' pressure.

This experiment shows that the method of alcoholic extraction employed did not remove all of the water-soluble B from the beans. The residue was still potent in promoting growth.

Preparation of Extract and Residue Used with Lots 954 A, Period 2, and 954 B, Period 2.—1,000 gm. of finely ground *raw* navy beans were extracted continuously with alcohol-free ether for 24½ hours. 13.19 gm. of material were extracted. The residue was freed from ether at room temperature and extracted with

acetone in three consecutive 6 hour periods changing the flask containing the extract each time. On distilling off the acetone, 3.77 gm. were found to have been removed by extraction in the first two 6 hour periods and 0.75 gm. in the last. They were taken up in a small amount of ether and evaporated on dextrin (95.5 gm.) giving a final weight of 100 gm. This extract was fed in the ration of Lot 954 B, Period 2. The residue (that which was insoluble in acetone) was later heated and fed in the ration of Lot 954 A, Periods 2 and 3.

Preparation of Extract and Residue Used in Chart 1, Lot 952 A, Period 2, Chart 3, Lot 951 A, Period 2, and Chart 4, Lot 951 B, Period 2.—1,000 gm. of finely ground *raw* navy beans were extracted with alcohol-free ether for 25 hours. Upon evaporating off the ether, 13.30 gm. of material were found to have been removed by the extraction.

The residue from the above extraction was freed from ether at room temperature and then extracted with 95 per cent alcohol in three consecutive 6 hour periods, using fresh or recovered solvent each time in order to minimize the possible destructive action of heat on the extract. 25.5 gm. of material were left as the extract of the first two 6 hour periods after distilling off the alcohol and 7.85 gm. from the last 6 hour period. These extracts were taken up in a little water and alcohol and then evaporated on dextrin and dried at 90–95° for a few hours. The dry weight was 128.35 gm. The residue from the extraction was freed from alcohol by heating at 90° for 3 hours. Later part of this residue was soaked in water and heated in the autoclave at 15 pounds' pressure for 40 minutes. This preparation (alcohol-insoluble) was fed to Lot 951 A, Chart 3, Period 2.

The 128.35 gm. of dried dextrin residue carrying the alcoholic extract from 1,000 gm. of *raw* navy beans was next extracted with acetone in a small Soxhlet apparatus, again in three consecutive 6 hour periods. After removing the acetone with minimum heat exposure, 7.96 gm. of extract were left from the first two 6 hour periods and 0.81 gm. from the last 6 hour period. They were taken up in alcohol, evaporated on dextrin, and made up to 100 gm. This mixture was fed to Lot 951 B, Chart 4, Period 2. The residue from the acetone extraction was freed from acetone and made up to 125 gm. with dextrin. It was employed in the ration of Lot 952 A, Chart 1, Period 2.

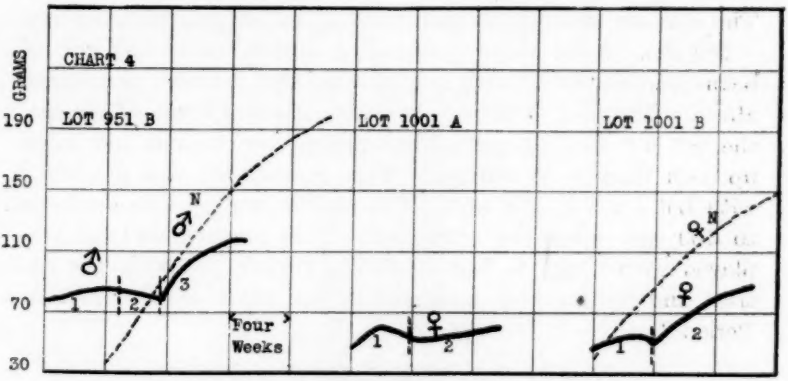
Chart 4. Lots 951 B, 1,001 A, and 1,001 B.

Lot 951 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with the acetone-soluble part of the alcohol-soluble part of ether-extracted *raw* navy beans, equivalent to 30 per cent of beans in the diet.

Period 3.—Ration: Same as Period 2 but with the addition of the acetone-insoluble part of the alcohol-soluble part of beans, equivalent to 30 per cent of beans; *i.e.*, the equivalent of the entire alcohol-soluble part of the beans, after being heated with acetone.

This experiment shows that even after extraction with alcohol, the water-soluble B is but slightly soluble in acetone. This conclusion is further supported by Chart 4, Lot 1,001 B, Period 2, and Chart 5, 1,001 C, Period 2.



In Chart 1, Lot 952 A, Period 2, it is shown that no growth could be secured with the alcohol-soluble, acetone-insoluble portion of beans equivalent to 30 per cent of beans in the diet. This suggests that there is a slow destruction of the water-soluble B during heating with acetone.

Lot 1,001 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus the acetone extract of the alcoholic extract and residue of same, of 10 gm. of ether-extracted *cooked* navy beans; *i.e.*, the equivalent of the alcoholic extract of navy beans after being heated with acetone.

Lot 1,001 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus the acetone-soluble part of the alcoholic extract of ether-extracted *cooked* navy beans equivalent to 60 gm. of beans.

This experiment shows that the water-soluble B is slightly soluble in acetone but very much less so than in benzene (compare with Chart 1, Lot 953 A, Period 3, and Chart 5, Lot 1,001 C, Period 2).

Preparation of Extract and Residue Employed with Lots 1,001 A, 1,001 B, and 1,001 C.—935 gm. of *cooked* navy beans were extracted with ether for 18 hours. The residue, freed from ether, was then extracted with alcohol for 18 hours in three consecutive 6 hour periods, changing the flask containing the extract at the end of each period. The first 12 hours' extract evaporated on dextrin gave 26.7 gm. of material, the last 6 hours' gave 7.6 gm. The extracts were united and made up to 200 gm. with dextrin.

100 gm. of the above preparation equivalent to 467 gm. of beans were extracted with freshly redistilled acetone, as outlined above. The first 12 hours' extraction gave 14.7 gm. of extract, the last 6 hours' 0.5 gm. The extracts were united and made up with dextrin to 100 gm. This preparation was employed with Lot 1,001 B, Period 2. The residue was likewise made up to 100 gm. after the extraction. This preparation was employed with Chart 5, Lot 1,001 C, Period 2. Both the extract and residue were employed in the ration of Lot 1,001 A, Period 2.

Chart 5. Lots 1,001 C, 836 R, and 836 E.

Lot 1,001 C. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 but with the residue of the acetone extract of the alcoholic extract of ether-extracted *cooked* navy beans equivalent to 60 per cent of beans; *i.e.*, the alcohol-soluble, acetone-insoluble part.

This experiment shows that in time hot acetone separates a part of the water-soluble B from the *cooked* navy beans (see Chart 4, Lot 1,001 B), but leaves the larger part of it in the residue. Rats grow on both the acetone extract and its residue when the plane of intake is sufficiently raised.

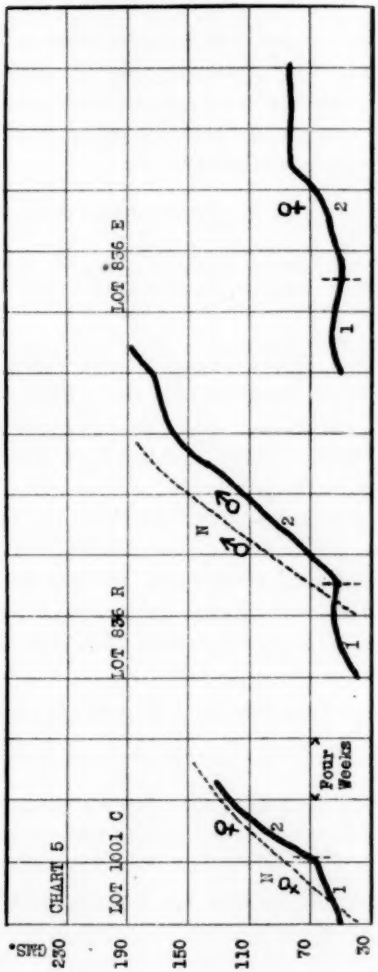


CHART 5
LOT 1001 C
LOT 836 R
LOT 836 E
GMS.
230
190
150
110
70
30
Four Weeks
N
1
2

Lot 836 R. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus 3 per cent of the residue after ether and acetone extraction of wheat embryo.

This experiment shows that acetone does not take out to any appreciable extent the water-soluble B from ether-extracted wheat embryo. Studies with pigeons indicate that traces are removed by this solvent, for the acetone extract of wheat embryo relieves polyneuritic pigeons (4).

Lot 836 E. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 but with the acetone extract of ether-extracted wheat embryo, equivalent to 14.28 per cent of wheat embryo in the diet.

This experiment shows that acetone takes out the water-soluble B from wheat embryo to a very slight extent.

Preparation of the Extract and Residue Employed with Chart 5, Lots 836 R and 836 E; Chart 6, Lot 836 R + E.—500 gm. of wheat embryo previously extracted with alcohol-free ether for 18 hours were extracted continuously with hot acetone in a Soxhlet apparatus for 18 hours. The acetone was distilled off, the residue taken up with hot water, and evaporated on 300 gm. of dry dextrin. After drying, 3.2 gm. were found to have been removed in the extraction process. The extract was made up to 400 gm. with dextrin.

Chart 6. Lots 836 R + E, 789 A, and 791 A.

Lot 836 R + E. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 but with the addition of 3 per cent of the ether- and acetone-extracted residue plus the acetone extract of 14 gm. of ether-extracted wheat embryo per 100 gm. of ration.

This experiment shows that the water-soluble B is not rapidly destroyed by prolonged treatment with hot acetone. There was no evidence from the appearance or activity that these rats receiving both the acetone extract and residue as in 836 R + E were in better condition than those receiving only the residue, as in Chart 5, Lot 836 R. This supports the view that there is but a *single substance* in what we term water-soluble B.

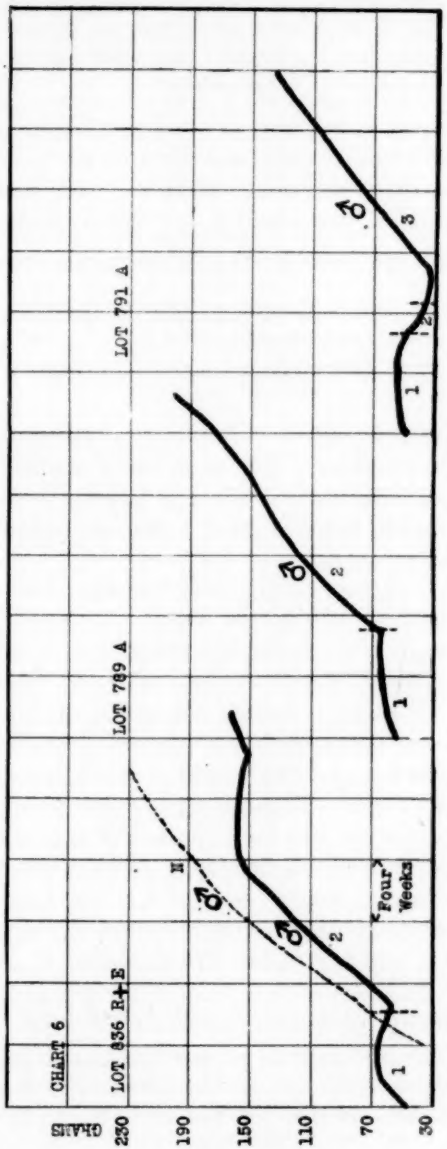


CHART 6
LOT 856 R+E
LOT 789 A
LOT 791 A
GRAMS
230
190
150
110
70
30
Four Weeks

Lot 789 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus 3 per cent of the ether- and benzene-extracted residue of wheat embryo.

This experiment shows that hot benzene does not remove the water-soluble B from ether-extracted wheat germ directly. This is in agreement with the results obtained with beans (Chart 1, Lot 953 A, Period 2, and Chart 2, Lot 994 A, Period 2).

Lot 791 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus the benzene extract of 14.28 per cent of ether-extracted wheat embryo.

Period 3.—Ration: Same as Period 2 plus 3 per cent of the ether- and benzene-extracted residue of wheat embryo.

Two rats had polyneuritis in Period 2. In Period 3 the ration caused prompt recovery. This experiment shows that benzene does not remove the water-soluble B directly from wheat embryo. The residue fed in Period 3 still contained the physiologically active substance.

Preparation of Extract and Residue Employed with Chart 6, Lots 789 A and 791 A.—1,000 gm. of wheat embryo which had been extracted 18 hours with alcohol-free ether were extracted with hot benzene continuously for 18 hours. Most of the benzene was then distilled off and the residue which was readily soluble in a little benzene was evaporated in a weighed crystallizing dish and dried *in vacuo* 24 hours. The weight of the material dissolved by benzene was 3.629 gm. This was again taken up in benzene and evaporated on dextrin, and made up to 200 gm. with dried dextrin. This preparation was employed in the ration of Lot 791 A, Period 2.

The benzene-insoluble material was used in the rations of Lot 789 A, Period 2, and also in Lot 791 A, Period 3.

Chart 7. Lots 955 A, 956 A, and 1,000 A.

Lot 955 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus the chloroform extract of ether-extracted wheat embryo equivalent to 15 per cent of wheat embryo.

Lot 956 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

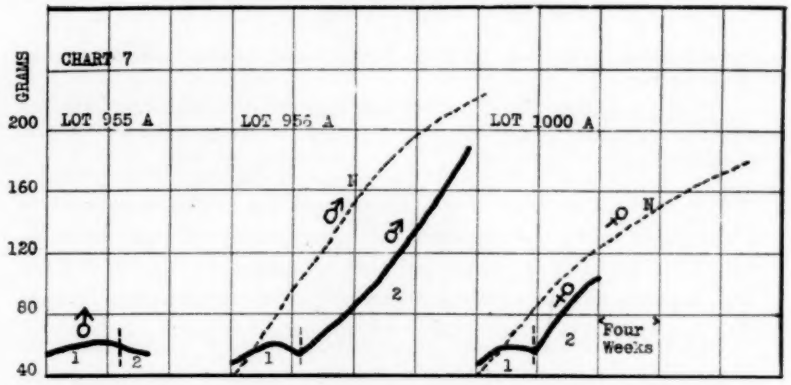
Period 2.—Ration: Same as Period 1 but with the addition of 3 per cent of wheat germ which had been extracted with ether and afterwards with hot chloroform for 18 hours.

This experiment shows that neither ether nor hot chloroform takes out the water-soluble B from wheat embryo.

Lot 1,000 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 but with the alcoholic extract of ether-extracted *raw* beans equivalent to 10 per cent of beans.

It has been shown previously that it is very difficult, if at all possible, to extract completely the water-soluble B from beans



by means of hot alcohol (Chart 3, Lot 951 A, Period 3). This is doubtless due to the great difficulty of grinding beans to an impalpable powder.

Preparation of the Extract and Residue Employed with Chart 7, Lots 955 A and 956 A.—589 gm. of wheat embryo previously extracted with ether for 18 hours were extracted with chloroform in three consecutive 6 hour periods. 0.838 gm. was extracted in the first two 6 hour periods and 0.218 gm. in the last. The extracts were taken up in chloroform, evaporated on dextrin, and made up to 58.9 gm. with dried dextrin. All evaporating and drying operations were carried out with the least possible exposure to heat. The extract (chloroform-soluble material) was used in the ration of Lot 955 A. The chloroform-extracted residue of wheat embryo was employed in the ration of Lot 956 A.

Preparation of the Extracts Employed with Chart 7, Lot 1,000 A and Chart 8, Lot 1,000 B.—1,052 gm. of *raw* navy beans after extraction with ether for 18 hours were extracted with hot alcohol for 18 hours in 3 consecutive 6 hour periods, changing the flasks containing the extracts at the end of each period. The first 12 hours' extraction yielded 45 gm. of extractives, the last 6 hours' 10.2 gm. The extracts were evaporated on dextrin, united, and made up to 200 gm. with dextrin. This extract was used in the ration of Chart 7, Lot 1,000 A, equivalent to 10 per cent of beans, and in Chart 8, Lot 1,000 B, equivalent to 15 per cent of beans.

Chart 8. Lots 1,000 B, 973 A, and 973 B.

Lot 1,000 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with the addition of the hot alcoholic extract of 15 gm. of ether-extracted *raw* navy beans per 100 gm. of ration.

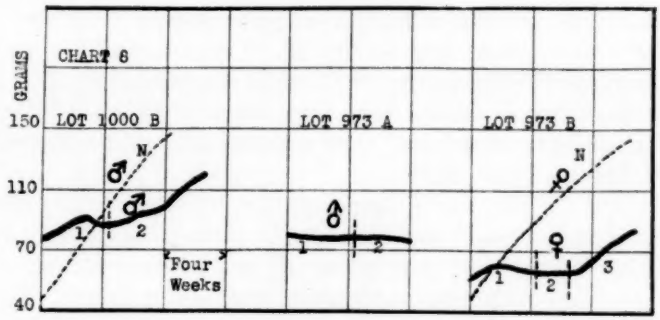
The animals in this lot which received the alcoholic extract of 15 per cent of *raw* beans were but little better off than those in Chart 7, Lot 1,000 A, which got their supply of the factor B from the alcoholic extract of 10 per cent of beans. Incomplete extraction appears to be unavoidable when beans are used.

Lot 973 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with the *residue* of the benzene extract of the alcoholic extract of 30 per cent of ether-extracted, *cooked* navy beans per 100 gm. of ration; *i.e.*, the alcohol-soluble, benzene-insoluble portion was fed.

At first thought this curve might give the impression that in the process of heating with water a progressive destruction of the water-soluble B takes place. 25 per cent of navy beans heated for 1¼ hours at 15 pounds' pressure still induces normal growth when combined with a suitable mixture of purified foodstuffs (5) plus 5 per cent of butter fat. In Chart 1, Lot 953 A, Period 3, it was shown that the benzene extract of the alcoholic extract of 30 per cent of ether-extracted *raw* navy beans sufficed to induce good growth. The benzene extract of the alcoholic ex-

tract of 30 gm. of ether-extracted *heated* beans did not in Lot 973 B suffice to furnish enough of the factor B to induce growth, but growth at a moderate rate took place when the equivalent of 60 gm. of beans per 100 gm. of ration was included. Chart 10, Lot 933, shows, however, that even 10 per cent of *cooked* navy beans suffices to induce normal growth during 7 weeks. There may be slight destruction of the water-soluble B during cooking of beans but destruction proceeds very slowly, if at all. The explanation of the failure of the extracts of *cooked* beans to support growth as well as extracts of similar amounts of *raw* beans, lies in the failure of alcohol to extract the water-soluble B



very efficiently from *cooked* beans. This is apparently due to change in the physical properties of the bean.

Lot 973 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with the benzene *extract* of the alcohol-soluble part of 30 gm. of ether-extracted *cooked* navy beans per 100 gm. of ration; *i.e.*, the alcohol-soluble, benzene-soluble portion was fed.

Period 3.—Ration: Same as in Period 2, but with the extract increased to the equivalent of 60 per cent of beans.

This experiment might seem to suggest that there is progressive destruction of the factor B on cooking the beans (compare Chart 1, Lot 953 A, Period 3). The benzene extract of the alcoholic extract of 30 gm. of *raw* beans, prepared in the same manner as the extract in this experiment, suffices for the promotion of growth. Chart 10, Lots 932 and 933, however, makes it evident

that the dietary unknown is not greatly reduced in amount by the heat treatment, for 10 per cent of *cooked* beans still supplies enough to support normal growth for 7 weeks. Rats do not eat rations which contain appreciable amounts of raw beans, so it is not possible to conduct comparable experiments with heated and unheated beans. The true explanation of these results is that apparently because of physical change in the beans during cooking alcohol fails to extract the water-soluble B after the heat treatment.

Preparation of Extracts Employed with Lots 973 A and 973 B.—918 gm. of navy beans (*cooked*) were extracted with ether for 18 hours and then with alcohol in three consecutive 6 hour periods, removing the extract at the end of each period. The first two extracts were evaporated on 100 gm. of dextrin and the alcohol was removed. There was an increase in weight of 37.1 gm. The last 6 hours' extract was evaporated on 50 gm. of dextrin giving 11.1 gm. of extract. The extracts were united and made up to 200 gm. with dextrin.

100 gm. of the above preparation equivalent to 459 gm. of beans were extracted with benzene in three consecutive 6 hour periods, removing the extract at the end of each period. The extracts were evaporated in weighed crystallizing dishes. Weight of first extract, 9.80 gm.; second extract, 0.025 gm.; third extract, trace. The extracts were taken up in benzene and evaporated on dextrin. Final weight 91.8 gm. The alcohol-soluble, benzene-insoluble portion was fed to Lot 973 A. The alcohol-soluble, benzene-soluble part was fed to Lot 973 B, first, equivalent to 30 and later raised to 60 gm. of beans per 100 gm. of ration.

Chart 9. Lots 999 A, 999 B, and 972 B.

Lot 999 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with the addition of the alcoholic extract of 10 gm. of ether-extracted *cooked* navy beans per 100 gm. of ration.

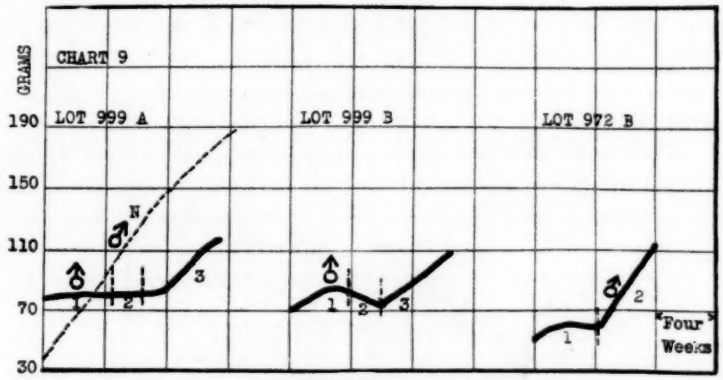
Period 3.—Ration: Same as Period 2, but with the extract increased to make the equivalent of 30 gm. of *cooked* beans per 100 gm. of ration.

Lot 999 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus the alcoholic extract of 15 gm. of ether-extracted *cooked* beans.

Period 3.—Ration: Same as Period 2, but with the extract increased to equal 40 per cent of beans in the food mixture.

This illustrates the difficulty of complete extraction of the dietary factor B from beans by alcohol after cooking. 10 per cent of beans furnish enough of this substance for normal growth during 7 weeks, but the alcoholic extraction is so incomplete when *cooked* beans are employed that the extracts used in this experiment were not adequate.



Lot 972 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with the *residue* of 25 per cent of *cooked* beans, after extraction with ether and then with alcohol.

This experiment supports the evidence afforded by Chart 9, Lot 999 A and by Lot 999 B, that the failure of the alcoholic extract of *cooked* beans to induce growth is due to physical changes in the bean during cooking whereby the water-soluble B is protected from contact with the solvent and not because of destruction of the physiologically active substance.

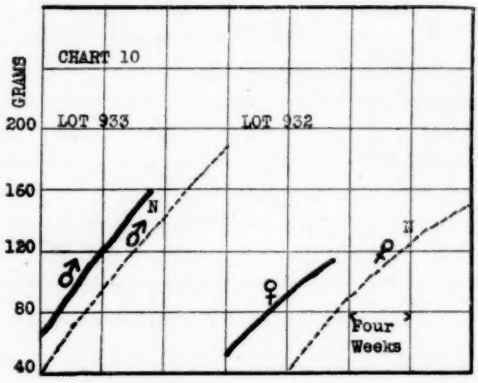
Preparation of the Extract and Residue Employed with the Rations of Chart 9, Lots 999 A, 999 B, and 972 B.—1,176 gm. of *cooked* navy beans were extracted with alcohol-free ether for 18 hours and then with 95 per cent alcohol in three consecutive 6 hour periods, removing the flask with the extract at the end of each period to prevent excessive heating. The first 12 hours' extrac-

tion removed 28.4 gm. of material, the last 6 hours' 11.9 gm. The extracts were united and made up to 200 gm. with dextrin. The alcohol-soluble material was fed to Lots 999 A, Period 2, and 999 B, Period 2; the alcohol-insoluble portion of the beans was fed to Lot 972 B, Chart 9, Period 2.

Chart 10. Lots 933 and 932.

Lot 933.—Ration: Purified food mixture plus 5 per cent of butter fat, plus 10 per cent of *cooked* navy beans as the sole source of the water-soluble B.

Lot 932.—Same as Lot 933, but with 5 per cent of *cooked* beans as the source of the dietary factor B.



These curves obtained with 10 and 5 per cent respectively of *cooked* navy beans point to the great richness of the navy bean in the dietary essential water-soluble B. They indicate that probably because of a change in the physical properties of the beans during cooking, alcohol fails to extract this substance as well as it does from *raw* beans.

The stability of the dietary factor B when heated with water is a matter of great importance in practical dietetics. The stability of the factor B to heat makes possible its isolation by methods involving a considerable amount of manipulation, provided the conditions are kept suitable.

The Solubility of the Water-Soluble B as It Exists in Animal Tissues.

In our studies of the fat-soluble A we have repeatedly observed that, while ether extracts of egg yolk and the ether-soluble constituents of milk contain the unidentified A, plant fats, isolated from the seed by means of ether apparently never contain this substance (18). It remains after exhaustive extraction, in the fat-free residue of the plant tissue. We have suggested that the substance (fat-soluble A) is in chemical union in the plant tissues in a form which is not soluble in ether, and that during digestion or absorption it is set free and, being readily soluble in fats (18), thereafter accompanies the fats in the animal body. It seemed desirable to extend our observations on the solubility of the water-soluble dietary essential in organic solvents to an animal tissue, since one cannot extend with confidence the results obtained with plant tissues to tissues of animal origin, owing to the possibility that it exists in the animal cell in chemical union. Our data on this subject is still meager but that obtained with dried, unheated pig kidney will be reported here.

Chart 11. Lots 962 A, 961 A, 961 B, and 960 B.

Lot 962 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 with the addition of 6 per cent of dry, uncooked pig kidney extracted with ether and then with benzene.

This experiment is in agreement with Lot 961 A, which shows that the physiologically active substance is not extracted to an appreciable extent from animal tissue (kidney) by ether or benzene.

Lot 961 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with the benzene extract of ether-extracted pig kidney equivalent to 6 gm. of tissue per 100 gm. of ration.

Period 3.—Ration: Same as Period 1, but with the addition of the benzene extract of the alcoholic extract of ether-extracted *raw* navy beans equivalent to 30 gm. of beans per 100 gm. of ration (the ration of Chart 1, Lot 953 A, Period 3).

The preparation from beans was introduced in Period 3 to demonstrate the capacity of the animals to grow.

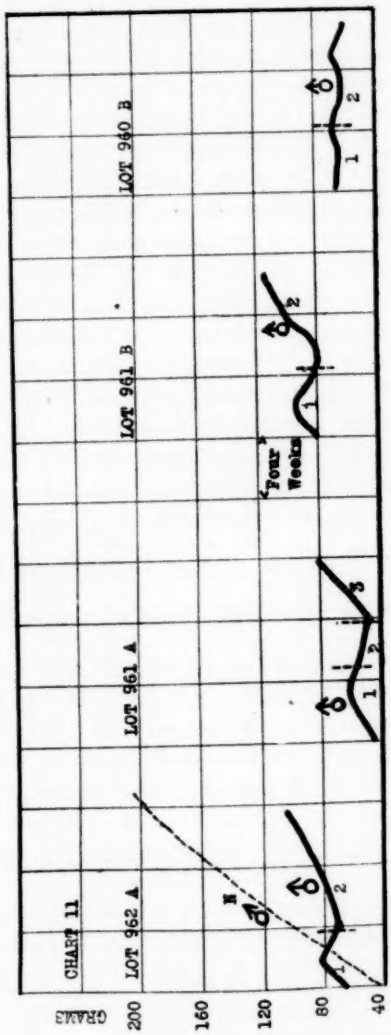


CHART 11
LOT 962 A
LOT 961 A
LOT 961 B
LOT 960 B
GRAMS
200
160
120
80
40
Four Weeks
1
2
3

Taken in connection with the results obtained in feeding the benzene extract of navy beans (Chart 1, Lot 953 A, Period 2), this experiment demonstrates the relative insolubility of the water-soluble B in hot benzene in animal tissues as well as in plant tissues.

Lot 961 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 but with 6 per cent of the residue after extraction of dry uncooked pig kidney with ether, then with acetone.

Dry, uncooked pig kidney behaves toward ether and acetone in the same way as do the navy bean and wheat germ, in that the water-soluble B is not readily extracted even by prolonged treatment with these solvents.

Lot 960 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with the addition of the acetone extract of ether-extracted pig kidney equivalent to 6 gm. per 100 gm. of ration.

This experiment shows when considered along with Lot 961 B, that acetone does not readily extract the dietary essential, water-soluble B, from dried raw pig kidney. That a small amount was extracted is indicated by the fact that the animals in this group failed to develop the loss of muscular control except in one case, and this was at the end of the 7th week after the addition of acetone extract of kidney, and at the end of the 11th week of the experiment. Animals on the ration of Period 1 never live as long as this, which shows that the acetone extract furnished a little of the water-soluble B. Lot 961 B (Chart 11) was able to make appreciable growth, and although undersized was apparently normal. After the acetone extraction, the greater part of the water-soluble B was still in the residue.

Preparation of the Extract and Residue Employed in the Ration of Lot 961 B and 960 B.—1,935 gm. of fresh pig kidney were ground up in a meat chopper and then dried at 95° for 24 hours. The weight of the dried kidney was 485 gm. which was equivalent to 25 per cent of the original weight of tissue. The dried residue was ground, then extracted with ether for 4 hours, and then ground up again and the extraction continued for an additional

14 hours. 25 per cent of ether-soluble material was thus removed, leaving 360 gm. of residue.

112.9 gm. of the above ether-free residue were extracted with acetone in three consecutive 6 hour periods, changing the flask at the end of each period. The first two 6 hour periods gave 4.572 gm. of extract, the last 6 hour period 2.26 gm. They were taken up in alcohol and evaporated on dextrin at 40°. The final weight of extracts on dextrin was 34.4 gm. This extract (acetone-soluble material) was fed in the ration of Lot 960 B, Period 2. The residue after extraction (acetone-insoluble material) was fed in the ration of Lot 961 B, Period 2.

Preparation of Extract and Residue Employed with Lots 961 A and 962 A Respectively.—122 gm. of ether-extracted kidney residue obtained as previously described were extracted with benzene in three consecutive 6 hour periods changing the flask with the extract at the end of each period. 4.10 gm. of extract were obtained in the first two 6 hour periods and 0.051 gm. in the last. Both fractions were united, and were then evaporated on dextrin yielding 28.15 gm. The benzene-soluble matter was fed to Lot 961 A, Period 2; the benzene-insoluble residue was fed to Lot 962 A, Period 2.

The Stability of the Water-Soluble B toward Nitrous Acid, Hydrochloric Acid, and Dilute Sodium Hydroxide.

The studies of Williams and his coworkers (3) and of Sullivan and Voegtlin (3) have indicated that the substance which cures polyneuritis in fowls is rapidly destroyed by even moderately dilute alkalies, but is relatively stable toward mineral acids. We have examined preparations of the water-soluble B prepared from wheat embryo by extraction with alcohol after removal of the fats by means of ether, for their power to induce growth in rats. The preparations, previous to feeding, were treated with one of the following reagents: (*a*) nitrous acid, (*b*) hydrochloric acid, (*c*) sodium hydroxide. Our results with alkalies are in agreement with those of Williams and Sullivan and Voegtlin.

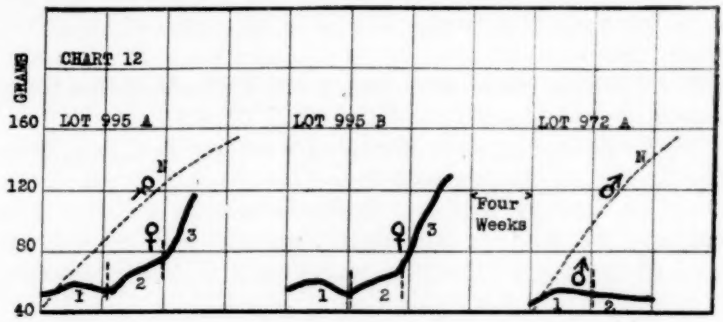
Chart 12. Lots 995 A, 995 B, and 972 A.

Lot 995 A. *Period 1.*—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus a preparation of water-soluble B treated 4 hours with nitrous acid. An extract equivalent to 3 per cent of wheat embryo was fed.

Period 3.—Ration: Same as Period 2, but with the water-soluble B preparation treated with nitrous acid increased to the equivalent of 10 per cent of wheat embryo.

It is possible that treatment with nitrous acid tends to destroy the dietary factor B to a slight extent, but it does so very slowly, if at all. The resistance of this substance to nitrous acid is a strong indication that it is neither a primary nor a secondary amine.



Lot 995 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with a preparation containing the water-soluble B equivalent to 3 per cent of wheat embryo in the food mixture. This preparation was evaporated with hydrochloric acid as described below.

Period 3.—Ration: Same as Period 2 with the water-soluble B preparation increased to the equivalent to 10 per cent of wheat embryo.

It is not clear from this experiment whether or not the treatment with hydrochloric acid destroyed a part of the dietary factor B. The fact that there was no growth in Period 3 would seem to indicate that the substance is slowly inactivated. This phase of the subject will receive further study. Relatively great stability toward hydrochloric acid is clearly shown.

Treatment of Preparations Employed with Lots 995 A and 995 B.—416 gm. of wheat embryo previously extracted with ether for 18 hours, were extracted with 95 per cent alcohol for 6 hours using the Soxhlet system and for three consecutive 6 hour periods using the Caldwell system. The alcohol was distilled off from each portion separately and the extracts were brought down to dryness in a large granite pan at 40–50° in 14 hours. The residue was taken up in water and made up to 416 cc.

208 cc., equivalent to 208 gm. of wheat embryo, were transferred to an aspirating bottle and the nitrous acid gas was generated from 50 gm. of sodium nitrite, and 100 cc. of 1:1 HCl were passed in during the course of 4 hours. The gas was well washed by passing it through a strong sodium nitrite solution. The treated solution was brought down and evaporated on dextrin. The total weight of dextrin carrying the extract was brought up to 208 gm. This extract was fed in the ration of Lot 995 A (Chart 12).

The remaining 208 cc. were treated with HCl as described below and employed in the ration of Lot 995 B (Chart 12).

The solution of alcoholic extract of wheat embryo (from Preparation 995 A) was acidified with 5 cc. of C.P. HCl and evaporated on dextrin. The dry residue was made up to a final weight of 208 gm. It was rather strongly acid, since 5 gm. required 25 cc. N/14 NaOH to neutralize it to phenolphthalein.

Lot 972 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 with 10 per cent of wheat embryo which had been treated with 0.28 per cent sodium hydroxide at 100°C. for 1 hour.

This treatment with dilute sodium hydroxide destroyed almost completely the content of water-soluble B in the wheat embryo. This confirms the observations of others that alkalies are very destructive of this dietary essential.

Preparation of the Wheat Embryo Treated with Alkali Which Was Fed to Lot 972 A, Period 2.—550 gm. of wheat embryo, previously ether-extracted, were extracted with 95 per cent alcohol for three consecutive 6 hour periods, the flask with the extract being removed at the end of each period to minimize the possible de-

structive action of heat. The alcohol was distilled off, the extract taken up in water, and made up to 550 cc. It was distinctly acid to litmus. It was neutralized to phenolphthalein with a sodium hydroxide solution and a sufficient excess added to make it alkaline up to 0.28 per cent NaOH. At this degree of alkalinity the solution was heated for 1 hour under a reflux, then cooled, then almost neutralized with hydrochloric acid, and acidified by the addition of 12 per cent acetic acid which made it strongly acid. It was evaporated on dextrin in large granite pans and dried at 70–80° for 5 hours.

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EFFECTS OF DIET ON THE PLASMA CHLORIDES AND CHLORIDE EXCRETION IN THE DOG.

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In the study of the plasma chlorides in a series of nephritics Wolferth (1) recently called attention to the low figures occasionally observed in certain cases. As a rule, this finding was noted in patients who had been for some time upon a chloride-free diet. This observation we (2) have confirmed and have found the plasma chlorides as low as 4.6 gm. per liter in one case of advanced glomerulonephritis, 5 days before death. That this marked depression of the plasma chlorides might be merely the result of a long continued low chloride diet combined with a rather free administration of water seemed to us possible in spite of the well recognized tendency of the blood to maintain constant its inorganic composition. It was observed that in other cases with chronic nephritis, especially those of the so called parenchymatous type, which exhibited a tendency to elevation of the plasma chlorides, even a prolonged period of low salt diet did not bring the plasma chlorides below normal or often even to the normal figure. These, however, do not afford satisfactory evidence of the effect of a continued low chloride diet on the plasma in other diseases of the kidneys or when the kidneys are normal.

In order to obtain some data upon the point the following experiments were carried out upon dogs. The animals were placed upon one of three diets; an ordinary diet, a diet with a high sodium chloride content, or a diet poor in sodium chloride and with a free administration of distilled water. After a vari-

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able number of days upon the diet, a period of from 20 to 24 hours was allowed to elapse after the last feeding and the dog was then catheterized and given 100 cc. of distilled water by stomach tube to promote diuresis. About 35 minutes later blood was taken from the jugular vein for chloride analysis, and about 70 minutes after the first catheterization the animal was catheterized again and the concentration of chlorides in the urine determined.

The rate of excretion of chlorides was calculated for a 24 hour period on the basis of the excretion during the interval between the catheterizations. This portion of each experiment is designated in the protocols and table by the letter "A" following the number of the experiment. The series of "A" experiments shows the condition of the plasma chlorides and of the chloride excretion as a result of the continued use of the various diets. The main object of this part of the investigation was to determine whether following several days upon a low chloride diet the plasma chlorides would be found at a lower level than following a period on ordinary diet, and also to determine whether following a series of days on high chloride diet the plasma chlorides would be found increased, and if so whether with or without an increased rate of chloride excretion in the urine. In order to determine whether any injury to the general renal function resulted from the high salt diet, blood urea was determined in certain of the experiments and the figures were considered as evidence of the general renal functional integrity.

In order to test the immediate effect of a large dose of sodium chloride upon the plasma and upon the chloride excretion a second procedure designated "B" in the protocols and table was carried out in many of the experiments. Immediately upon completion of part "A" of the experiment 10 gm. of sodium chloride in two doses an hour apart were given by stomach tube in 5 per cent solution. Immediately after the second dose a period of urinary collection was instituted as in "A" and blood again taken for analysis in the middle of this period.

In these experiments can be noted: first, the relation between the increase in the plasma chlorides and the increase in the chloride excretion resulting from the administration of the sodium chloride; second, the effect of the previous dietary régime upon this response to the dose of chloride.

In two instances on the day following the administration of the 10 gm. of sodium chloride just described a third period of urinary collection with collection of blood was carried out to determine whether any effect of the large dose of chloride still persisted 24 hours later, either in the plasma or in the rate of chloride excretion. This part of the experiment is designated in the protocols and table as "C."

In Experiment 4, Dog I, the effect of a mild uranium nephritis in an animal on a medium chloride diet was studied for comparison with the effect of high salt feeding.

Methods.

Urine.—The chlorides were estimated by the Volhard-Harvey method (3). The concentration is indicated in the protocols and table as gm. of sodium chloride per liter of urine under "E." The rate of chloride excretion is expressed as gm. of sodium per 24 hours, calculated from the excretion in the number of minutes of urinary collection employed in each experiment; this rate of excretion is given in the protocols and table as "D."

Blood.—Blood was drawn from the jugular vein directly into a centrifuge tube containing potassium oxalate crystals beneath paraffin oil, centrifuged immediately, and the plasma pipetted from the cells (4). In the plasma so obtained chlorides were estimated by the method of McLean and Van Slyke (5). The figures are given under "N" as gm. of sodium chloride per liter of plasma.

Blood urea nitrogen was estimated on the whole blood by the method of Van Slyke and Cullen (6). The figures given are mg. of blood urea nitrogen per 100 cc. of blood. In some of the experiments blood was also drawn from the vein, allowed to clot, centrifuged, and the serum used for the estimation of electrical conductivity. Decinormal potassium chloride solution was used as a standard and determinations were made at 25°C. The specific conductivity is given under "X."

In all analyses duplicate determinations were made.

RESULTS.

The results of the fourteen experiments on the three dogs are given in the table. The detailed protocols are appended.

It will be noted that on the same ordinary régime the three dogs differed somewhat in the level of plasma chlorides; Dog I showing the highest, 6.3 gm. per liter, and Dog II the lowest, 5.9 gm. The chloride excretion in all three was small.

On a high salt diet consisting of ordinary diet plus from 5 to 10 gm. of sodium chloride daily continued from 4 to 13 days Dogs I and III showed a definite, although in the case of Dog III a very slight, increase in the level of the plasma chlorides. The study of the blood and urine was made the day following the last feeding and last dose of chloride. The urinary excretion of chloride was still definitely increased in Dog III but was normal in Dog I. Dog II developed a marked diarrhea as soon as the chloride high diet was commenced and this probably accounts for the absence of any increase in the chloride excretion and for the very slight degree of change in the plasma chlorides the day following the last dose of chloride.

In Experiment 14, 48 hours were allowed to elapse between the last dose of chloride and the study of the blood and urine, and this interval proved sufficient to allow a return of the plasma chlorides and chloride excretion to normal, in spite of the fact that the dog had received 7.5 gm. of added sodium chloride daily for 20 consecutive days.

It seems, therefore, that such increases in the plasma chlorides and chloride excretion as were observed in "A" group of experiments 24 hours after the last day of high chloride diet represent merely the persistence of the acute disturbance of these factors which follow each administration of chloride as shown in the "B" group of experiments about to be discussed and which may be seen persisting into the following day in Experiment 7, "C." There is no evidence that the continued use of a high chloride diet leads to a permanent or stable increase in the plasma chlorides or to an elevation of the chloride threshold.

The use of a low chloride diet combined with a free flushing with distilled water failed to reduce the level of the plasma chlorides below normal. In no case were we able to reduce the plasma chlorides below the original level observed in that animal. The same constancy was observed in the electrical conductivity of the serum. These experiments indicate, therefore, that in those cases of clinical nephritis, in which following the use of a low chloride diet the plasma chlorides fall distinctly below normal, this depression of the plasma chlorides is evidence of disease and not merely a consequence of the patient's régime.

In the "B" group of experiments we studied the immediate

effect of administration of 10 gm. of sodium chloride by the stomach in two doses an hour apart upon the plasma chlorides, electrical conductivity of the serum, and rate of chloride excretion. A marked rise in the plasma chlorides resulted, amounting to from 0.5 to 1 gm. per liter. This was associated with a markedly increased chloride output in the urine. Inspection of the table shows that no formula could be applied which would express the relations between the increases in these two factors either with or without inclusion of the urinary chloride concentration in the formula. In Dog I the highest chloride output, absolute and percentile, was associated with the lowest plasma chloride observed in this animal in any of the examinations immediately after chloride administration.

The electrical conductivity of the serum exhibited an increase about commensurate with that of the plasma chlorides. In Dog III, Experiment 7, when the dog had been on a diet of moderate chloride content neither the plasma nor the urinary excretion had returned to normal on the day following the administration of 10 gm. of sodium chloride (Experiment 7, "C"). In Experiment 9, however, when the dog had been on a low chloride diet, the plasma chlorides had returned to normal the day following the administration of 10 gm. of sodium chloride, although the urine still showed a somewhat increased output.

In Experiment 4, in which the effect of a mild uranium nephritis was studied, the elevation of the plasma chlorides on an ordinary diet was very marked and the chloride excretion poor. Following the administration of 10 gm. of sodium chloride by stomach, although only a moderate increase occurred in the plasma chlorides, they reached the highest figure observed in any of these experiments, but almost no response resulted in the urinary chloride excretion. The behavior of the kidney in this experiment can clearly not be interpreted as due merely to the elevation of its threshold for chlorides as the result of the nephritis. Obviously in this experiment a given increment in the level of the plasma chlorides excited much less increase in the rate of the chloride excretion than was the case in the other experiments in which the kidneys were normal.

McLean (7) has shown that in the normal animal the chloride threshold is constant and that a definite rise in plasma chlorides

provokes an increment in urinary excretion proportional to the square of the rise. The constancy of this threshold even under a prolonged régime of extremely high or extremely low chloride diet is again demonstrated in our experiments. In the pathological kidney, however, there may be either an alteration in the threshold or a disturbance in the degree of renal response to increments in the plasma chlorides above the threshold. Because of these two variables interpretation of the significance of alterations in the chloride index in pathological cases is complicated.

PROTOCOLS.

Dog I.—Female, weight 14 kilos.

Experiment 1.—Ordinary diet. On a mixed diet of table scraps. Water *ad libitum.*

Experiment 1 A.—No food or water since previous day.

Sept. 28, 11.40 a.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 12.15 p.m. Bled 12 cc. 12.40 p.m. Catheterized. Only 1 cc. of urine obtained. Given 100 cc. of distilled water. 1.55 p.m. Catheterized. 160 cc. of urine (135 minutes). N, 6.3; D, 0.85; E, 0.5.

Experiment 1 B.—Sept. 28. 1.55 p.m. Given 100 cc. of 3 per cent sodium chloride solution by stomach tube. 3.00 p.m. Given 100 cc. of 5 per cent sodium chloride solution by stomach tube. Catheterized. 3.25 p.m. Bled 24 cc. 4.20 p.m. Catheterized. 135 cc. of urine (80 minutes). N, 7.3; D, 34.5; E, 14.2.

Experiment 2.—High salt diet. Dog placed for 5 days (Sept. 29 to Oct. 3) on a diet of milk, 1 quart, and one dog biscuit each day. In addition given each day (except Oct. 2) 150 cc. of 5 per cent sodium chloride solution by stomach tube. Water *ad libitum.*

Experiment 2 A.—No food, water, or salt since previous day.

Oct. 4, 2.45 p.m. Catheterized. Given 90 cc. of distilled water by stomach tube. 3.18 p.m. Bled 24 cc. 3.45 p.m. Catheterized. 15 cc. of urine (60 minutes). N, 6.7; D, 0.36; E, 1.

Experiment 2 B.—Oct. 4, 3.45 p.m. Given 95 cc. of 5 per cent sodium chloride solution by stomach tube. 4.43 p.m. Same dose of sodium chloride repeated. Catheterized. 5.20 p.m. Bled 24 cc. 5.45 p.m. Catheterized. 140 cc. of urine (62 minutes). N, 7.4; D, 49.5; E, 15.2.

Experiment 3.—Low salt diet. For 10 days (Oct. 18 to Oct. 27) on a low salt diet. (Milk, 1 quart, and dried salt-free bread.) In addition given each day 150 cc. of distilled water by stomach tube.

Experiment 3 A.—No food or water since previous day.

Oct. 28, 9.45 a.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 10.20 a.m. Bled 16 cc. 10.50 a.m. Catheterized. 12 cc. of urine (65 minutes). N, 6.3; D, 0.1; E, 0.4.

Experiment 3 B.—Oct. 28, 10.50 a.m. Given 100 cc. of 5 per cent sodium chloride solution by stomach tube. 11.55 a.m. Same dose of sodium chloride repeated. Catheterized. 12.30 p.m. Bled 20 cc. 1.05 p.m. Catheterized. 167 cc. of urine (70 minutes). N, 6.8; D, 53.3; E, 15.5.

Experiment 4.—Uranium nephritis. For 5 days (Oct. 29 to Nov. 3) on diet of milk, 1 quart, and one dog biscuit daily. Water *ad libitum.*

Oct. 30. Given uranium nitrate, 0.02 gm. subcutaneously.

Nov. 4. Shows a moderate cloud of albumin in urine.

Experiment 4 A.—No food or water since previous day.

Nov. 4, 12.03 p.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 12.37 p.m. Bled 12 cc. 1.25 p.m. Catheterized. 25 cc. of urine (82 minutes). N, 7.4; D, 0.13; E, 0.3.

Experiment 4 B.—Nov. 4, 1.25 p.m. Given 100 cc. of 5 per cent sodium chloride solution by stomach tube. 2.32 p.m. Same dose of sodium chloride repeated. Catheterized. 3.10 p.m. Bled 24 cc. 3.42 p.m. Catheterized. 35 cc. of urine (70 minutes). N, 7.7; D, 0.78; E, 1.04; blood urea N, 39.

Dog II.—Female, weight 16 kilos.

Experiment 5.—Ordinary diet. Diet of milk, 1 quart, and one dog biscuit daily beginning Dec. 27. Water *ad libitum.*

Experiment 5 A.—No food or water since previous day. Dec. 29, 11.07 a.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 11.52 a.m. Bled 25 cc. 12.25 p.m. Catheterized. 25 cc. of urine (78 minutes). N, 5.9; D, 0.67; E, 1.46; X, 0.0129; blood urea N, 14.

Experiment 5 B.—Dec. 29, 12.25 p.m. Given 100 cc. of 5 per cent sodium chloride solution by stomach tube. 1.50 p.m. Same dose of sodium chloride repeated. Catheterized. 2.32 p.m. Bled 35 cc. 3.10 p.m. Catheterized. 59 cc. of urine (80 minutes). N, 6.7; D, 17.1; E, 16.1; X, 0.0142.

Experiment 6.—High salt diet. Dog continued on the same diet of milk and dog biscuit. For 4 days (Jan. 2 to 5) received in addition sodium chloride as follows: Jan. 2 and 3, 130 cc. of 5 per cent sodium chloride solution by stomach tube; Jan. 4 and 5, 200 cc. of same. Water *ad libitum.* Diarrhea beginning Jan. 3.

Experiment 6 A.—No food, water, or salt since previous day.

Jan. 6, 11.00 a.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 11.35 a.m. Bled 30 cc. 12.25 p.m. Catheterized. 9.5 cc. of urine (85 minutes). N, 6.0; D, 0.56; E, 3.5; blood urea N, 10.

Dog III.—Female, weight 14 kilos.

Experiment 7.—Ordinary diet. Placed on a diet of milk, 1 quart, and one dog biscuit, beginning Dec. 27. Water *ad libitum.*

Experiment 7 A.—No food or water since previous day.

Jan. 2, 11.40 a.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 12.25 p.m. Bled 20 cc. 1.10 p.m. Catheterized. 5 cc. of urine (90 minutes). N, 6.0; D, 0.1; E, 1.2; X, 0.0132.

Experiment 7 B.—Jan. 2, 1.10 p.m. Given 100 cc. of 5 per cent sodium chloride solution by stomach tube. 3.00 p.m. Same dose of sodium chloride repeated. Catheterized. 3.35 p.m. Bled 30 cc. 4.10 p.m. Catheterized. 32 cc. of urine (70 minutes). N, 7.0; D, 14.6; E, 22.1; X, 0.0147; blood urea N, 10.

Experiment 7 C.—Received milk, 1 quart, one dog biscuit, and water *ad libitum* at 4.30 p.m. on Jan. 2. No further food or water.

Jan. 3, 10.20 a.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 11.30 a.m. Bled 12 cc. 12.20 p.m. Catheterized. 76 cc. of urine (120 minutes). N, 6.3; D, 21.5; E, 23.6.

Experiment 8.—High salt diet. In addition to diet of milk and dog biscuit as above, received daily 100 cc. of 5 per cent sodium chloride solution by stomach tube. Jan. 3 to 12. Water *ad libitum.*

Experiment 8 A.—Jan. 13. No food or water. 2.18 p.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 2.50 p.m. Bled 30 cc. 3.22 p.m. Catheterized. 23 cc. of urine (66 minutes). N, 6.2; D, 2.8; E, 5.7; X, 0.0133.

Experiment 8 B.—Jan. 13, 3.22 p.m. Given 100 cc. of 5 per cent sodium chloride solution by stomach tube. 4.05 p.m. Same dose of sodium chloride repeated. Catheterized. 4.32 p.m. Bled 20 cc. 5.20 p.m. Catheterized. 73 cc. of urine (75 minutes). N, 7.2; D, 27; E, 19.3; X, 0.0148.

Experiment 9.—Low salt diet. For 8 days (Jan. 18 to 25) given daily milk, 1 quart, and dried salt-free bread. In addition given daily 200 cc. of distilled water by stomach tube. Water *ad libitum,* but not taken.

Experiment 9 A.—No food or water since previous day.

Jan. 26, 1.32 p.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 2.05 p.m. Bled 30 cc. 2.35 p.m. Catheterized. 9 cc. of urine (63 minutes). N, 6.1; D, 0.4; E, 2.1; blood urea N, 10.

Experiment 9 B.—Jan. 26, 2.35 p.m. Given 100 cc. of 5 per cent sodium chloride solution by stomach tube. 3.25 p.m. Same dose of sodium chloride repeated. Catheterized. 3.55 p.m. Bled 20 cc. 4.45 p.m. Catheterized. 90 cc. of urine (80 minutes). N, 7.0; D, 25.3; E, 15.6.

Experiment 9 C.—Received milk, 1 quart, and salt-free bread at 5 p.m., Jan. 26. Water *ad libitum.* No further food or water.

Jan. 27, 9.35 a.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 10.15 a.m. Bled 20 cc. 10.45 a.m. Catheterized. 27 cc. of urine (70 minutes). N, 6.0; D, 1.2; E, 2.1.

Experiment 10.—Low salt diet. Same diet and distilled water as in Experiment 9 (Jan. 27 to Feb. 2).

Experiment 10 A.—No food or water since previous day.

Feb. 3, 2.42 p.m. Bled (No. 1) 20 cc. Catheterized. Given 100 cc. of distilled water by stomach tube. 3.30 p.m. Bled (No. 2) 30 cc. 4.07 p.m. Catheterized. 42 cc. of urine (85 minutes). No. 1: N, 6.1; X, 0.0133. No. 2: N, 6.0; D, 0.3; E, 0.4; X, 0.0132.

Experiment 11.—Low salt diet. Same diet and distilled water as in Experiments 9 and 10 (Feb. 5 to 11).

Dog.	Experiment No.	Diet.	No. of days on diet.	A. Before chloride administration.				Blood urea N.	B. After chloride administration.				C. 24 hrs. later.			
				N.	D.	E.	X.		N.	D.	E.	X.	N.	D.	E.	X.
				gm.	gm.	gm.			gm.	gm.	gm.		gm.	gm.	gm.	
I	1	Ordinary.	—	6.3	0.85	0.5	—	—	7.3	34.5	14.2	—	—	—	—	—
	2	High salt.	5	6.7	0.36	1.0	—	—	7.4	49.5	15.2	—	—	—	—	—
	3	Low "	10	6.3	0.10	0.4	—	—	6.8	53.3	15.5	—	—	—	—	—
	4	Uranium.	5	7.4	0.13	0.3	—	39	7.7	0.78	1.04	—	—	—	—	—
II	5	Ordinary.	—	5.9	0.67	1.46	0.0129	14	6.7	17.1	16.1	0.0142	—	—	—	—
	6	High salt (diarrhea).	4	6.0	0.56	3.5	—	10	—	—	—	—	—	—	—	—
III	7	Ordinary.	—	6.0	0.10	1.2	0.0132	10	7.0	14.6	22.1	0.0147	6.3	21.5	23.6	—
	8	High salt.	10	6.2	2.80	5.7	0.0133	—	7.2	27.0	19.3	0.0148	—	—	—	—
	9	Low "	8	6.1	0.40	2.1	—	10	7.0	25.3	15.6	—	6.0	1.2	2.1	—
	10	" "	7	6.0	0.30	0.4	0.0132	—	—	—	—	—	—	—	—	—
	11	" "	7	6.2	0.60	1.2	0.0133	14	—	—	—	—	—	—	—	—
	12	" "	8	6.2	0.36	0.6	0.0133	—	—	—	—	—	—	—	—	—
	13	High "	13	6.35	1.50	5.8	0.0134	14	—	—	—	—	—	—	—	—
	14	" "	6	6.2	0.50	0.7	—	13	—	—	—	—	—	—	—	—

N = gm. of sodium chloride per liter of plasma.
D = " " " " " day in urine (calculated).
E = " " " " " liter in urine.
X = Specific conductivity of serum.

Experiment 11 A.—No food or water since previous day.

Feb. 12, 3.00 p.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 3.45 p.m. Bled 30 cc. 4.30 p.m. Catheterized. 30 cc. of urine (90 minutes). N, 6.2; D, 0.6; E, 1.2; X, 0.0133; blood urea N, 14.

Experiment 12.—Low salt diet. Same diet and distilled water as in Experiments 9, 10, and 11 (Feb. 13 to 20).

Experiment 12 A.—No food or water since previous day.

Feb. 21. 3.12 p.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 3.52 p.m. Bled 25 cc. 4.37 p.m. Catheterized. 37 cc. of urine (85 minutes). N, 6.2; D, 0.36; E, 0.6; X, 0.0133.

Experiment 13.—High salt diet. Dog placed 13 days (Feb. 28 to Mar. 12) on milk, 1 quart, and one dog biscuit daily. Given 150 cc. of 5 per cent sodium chloride solution daily by stomach tube. Water *ad libitum.*

Experiment 13 A.—No food, water, or salt since previous day.

Mar. 13, 3.25 p.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 4.05 p.m. Bled 30 cc. 4.40 p.m. Catheterized. 13 cc. of urine (75 minutes). N, 6.35; D, 1.5; E, 5.8; X, 0.0134, blood urea N, 14.

Experiment 14.—High salt diet. Same diet and sodium chloride as in Experiment 13 (Mar. 14 to 19). Same diet, no sodium chloride, Mar. 20.

Experiment 14 A.—No food or water since previous day.

Mar. 21, 2.00 p.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 2.35 p.m. Bled 30 cc. 3.05 p.m. Catheterized. 32 cc. of urine (65 minutes). N, 6.2; D, 0.5; E, 0.7; blood urea N, 13.

CONCLUSIONS.

1. The level of plasma sodium chloride 24 hours after feeding varied in three dogs from 5.9 to 6.3 gm. per liter, but was quite constant for any one animal.

2. By administering large amounts of sodium chloride in solution by stomach tube, the level of the plasma chlorides can be raised in the dog within 1½ hours, 1 gm. per liter. The return to the previous level occurs within from 24 to 48 hours.

3. It is not possible to raise the level of the plasma chlorides other than in this transient manner by a prolonged régime on a high chloride diet, or to lower the plasma chlorides below the normal level even by a prolonged régime on a very low chloride diet.

4. In certain nephritic animals with impaired chloride excretion, whatever may be the hypothetical chloride threshold, a given increment in the plasma chloride concentration excites less increment in the rate of chloride excretion than it would in a normal animal.

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STUDIES OF ACID PRODUCTION.

II. THE MINERAL LOSS DURING ACIDOSIS.*

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The effect of acid administration and increased acid production on the excretion of certain minerals and nitrogen has been repeatedly studied. Recent discussions of the literature and bibliographies will be found in the publications of Forbes and Keith,[1] Givens and Mendel,[2] and Stehle.[3]

A survey of the literature failed to show a record of a complete experiment on the normal growing human organism. As children are peculiarly prone to acidosis it appeared desirable to supply the missing data. It was with this object in view that the following experiments were undertaken.

Methods.

The observations were made in a metabolism ward on two male children aged 5 and 8 years, and weighing 22.7 and 23 kilos, respectively. The boys were under the supervision of a special nurse. The food was prepared by a trained dietitian. Each period of observation lasted 3 days.

Each child consumed 436 gm. of egg white, 74 gm. of egg yolk, 414 gm. of milk, 64 gm. of pure sucrose, 60 gm. of pure lactose, 2 gm. of pure sodium chloride, and 3 gm. of agar, daily during the normal period. This amount of food contained 324 protein, 385 fat, 593 carbohydrate calories, and 12.6 gm. of nitrogen. The energy intake was equivalent to 56 calories per kilo. During the period of high fat feeding the protein calories remained the same, but the carbohydrate calories were reduced to 85,

* The expenses of the research were met in part by a grant from the research fund of the Graduate College of the State University of Iowa.

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while the fat calories were increased to 857. This was accomplished, by replacing the lactose and sucrose of the normal period, with an isodynamic quantity of pure butter fat (51 gm.) prepared according to the directions of Osborne and Mendel.[4] The mineral, calorie, and nitrogen intake, therefore, was practically identical during both periods.

The nitrogen and mineral content of the food was determined by analysis. The figures were compared with those obtained by calculation from the tables of Rose[5] (see Table I).

The urine of each voiding was separately collected in a Mason jar and preserved in the refrigerator after the addition of toluene. The 24 hour volume was diluted to 1 liter and 100 cc. were removed for the determination of titratable acidity, ammonia, creatine, creatinine, and acetone. The remaining urine of the 3 days (2,700 cc.) was united and analyzed for nitrogen and minerals. The feces were separated with the aid of carmine. The latter was administered, with the agar and milk, without other food, thus securing a sharp separation of the periods. The feces of a period were triturated with distilled water until a homogeneous suspension was obtained. Weighed aliquot portions were analyzed. The determinations of the minerals in the food, urine, and feces were carried out as described in a former article.[6] The sulfur of the food and feces was conveniently determined by the Benedict method, after treating the material with fuming nitric acid in a Kjeldahl flask. The modified oxidizing solution, recommended by Denis,[7] was found to be preferable to the original.

A record of the analytical data and results will be found in the following tables.

TABLE I.

*The Nitrogen and Mineral Content of the Daily Food.**

	Nitrogen.	Sulfur.	Phosphorus.	Chlorine.	Calcium.	Magnesium.	Sodium.	Potassium.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Calculated, Rose[5] . . .	12.6	1.11	0.7688	2.44	0.6496	0.0958	1.73	1.357
Found.								
Normal period	12.06	1.13	0.8799	2.31	0.7024	0.1465	1.781	1.261
High fat "	11.93	1.11	0.8631	2.49	0.6430	0.1046	1.762	1.207

* The addition of agar to the food probably accounts for the disagreement between the calculated and found values for calcium, magnesium, and phosphorus.

[4] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913–14, xvi, 423.

[5] Rose, M. S., A Laboratory Handbook for Dietetics, New York, 1912, 109.

[6] Baumann, L., and Howard, C. P., *Arch. Int. Med.*, 1912, ix, 665.

[7] Denis, W., *J. Biol. Chem.*, 1910–11, viii, 401.

TABLE II.

Record of the Excretion of Certain Blood and Urine Constituents during Periods of Normal and High Fat Feeding.

Day.	Diet.	Titratable acid.*	Ammonia.*	Creatinine.	Creatine.	Acetone (qualitative).	Carbonate of plasma.	Remarks.
		cc.	*cc.*	*mg.*	*mg.*		*vol. per cent*	
1	Normal.	125	385	276	192			Subject C. G., aged 5 yrs.;
2	"	135	385	274	194			weight 22.7 kg. Physi-
3	"	120	395	274	174		50	cal examination, nega-
								tive.
4	High fat.	140	330	272	178	+		During the high fat pe-
5	" "	170	500	256	236	++		riod, restlessness and a
6	" "	240	815	244	290	+++	29	slight increase of the
7	Normal.		818	284	202			pulse rate were ob-
								served.
1	Normal.	100	465	332	96			Subject R. W., aged 8
2	"	110	455	362	86			yrs.; weight 23.2 kg.
3	"	100	465	336	143		49	Physical examination
4	High fat.	120	390	362	124	+		showed scars of mas-
5	" "	120	425	342	216	++		toid and tonsillectomy
6	" "	145	530	334	250	+++	37	operations.
7	Normal.		630	361	297			

* The titratable acid and ammonia are expressed in terms of 0.1 N solution.

TABLE III.

The Intake, Output, and Balance of Minerals and Nitrogen during 3 Day Periods of Normal and High Fat Feeding.

		Nitrogen.	Sulfur.	Chlorine.	Phosphorus.	Sodium.	Potassium.	Calcium.	Magnesium.
C. G. Normal diet.	Food	36.18	3.399	6.930	2.6397	5.343	3.783	2.1072	0.4359
	Urine	34.17	3.27	7.242	2.019	5.100	3.528	0.8334	0.210
	Feces	0.156	0.1671	0.099	0.6671	0.0873	0.561	1.203	0.1503
	Balance	+1.845	−0.0383	−0.411	−0.0537	+0.1657	−0.306	+0.0708	+0.0792
C. G. High fat diet.	Food	35.79	3.33	7.458	2.5893	5.286	3.621	1.929	0.3138
	Urine	38.64	3.51	7.029	2.6055	5.724	4.260	1.3158	0.300
	Feces	2.145	0.2931	0.066	0.99	0.0609	0.7983	1.815	0.2799
	Balance	−4.975	−0.473	+0.363	−1.0057	−0.4989	−1.4373	−1.2018	−0.2661
R. W. Normal diet.	Food	36.18	3.399	6.93	2.6397	5.343	3.783	2.1072	0.4359
	Urine	35.76	3.345	7.029	1.965	5.082	3.147	0.4461	0.2424
	Feces	1.4565	0.216	0.0182	1.0191	0.0288	0.6411	1.7100	0.1452
	Balance	−1.03	−0.162	−0.1172	−0.3423	+0.2322	−0.0051	−0.0488	+0.0483
R. W. High fat diet	Food	35.79	3.33	7.458	2.5893	5.286	3.621	1.929	0.3138
	Urine	41.28	3.693	7.794	2.472	6.396	3.711	0.6966	0.3018
	Feces	1.107	0.1182	0.0156	0.8979	0.0369	0.9189	1.1230	0.3939
	Balance	−6.597	−0.481	−0.3512	−0.7806	−1.1460	−1.0889	+0.1084	−0.3819

TABLE IV.

The Mineral Excretion Expressed in Cc. 0.1 N Solution.

		Sulfur.	Phosphorus.†	Chlorine.	Calcium.	Magnesium.	Sodium.	Potassium.	Ammonia.
C. G. Normal diet.	Urine.*	1,853	1,302	2,042	416	203	2,217	902	1,165
	Feces..	104	430	28	600	124	38	143	
	Total..	1,957	1,732	2,070	1,016	327	2,255	1,045	
	Total acid....		5,759						
	" base....		5,808						
C. G. High fat diet.	Urine..	2,072	1,681	1,982	656	247	2,489	1,090	1,645
	Feces..	183	644	19	905	230	27	204	
	Total..	2,255	2,325	2,001	1,561	477	2,516	1,294	
	Total acid....		6,581						
	" base....		7,493						
R. W. Normal diet.	Urine..	1,983	1,268	1,982	223	199	2,209	805	1,385
	Feces..	138	657	5	853	119	13	164	
	Total..	2,121	1,925	1,987	1,076	318	2,222	969	
	Total acid....		6,033						
	" base....		5,970						
R. W. High fat diet.	Urine..	2,233	1,593	2,198	348	248	2,781	949	1,345
	Feces..	74	579	4	560	324	16	235	
	Total..	2,307	2,172	2,202	908	572	2,797	1,184	
	Total acid....		6,681						
	" base....		6,806						

* The acid equivalent of the total sulfates only is recorded.

† Phosphoric acid was calculated as a dibasic acid.

DISCUSSION.

Inspection of the tables shows that C. G., the younger child, responded promptly to the high fat diet with a marked increase in nitrogen, minerals, ammonia, titratable acid, and creatine.

During the preliminary normal period he excreted the equivalent of 5,759 cc. of mineral acid and 5,808 cc. of base; that is, 49 cc. of base served for the neutralization of organic acid. The titratable acidity, 380 cc., plus 49 cc. indicates the approximate organic acid excretion (429 cc.). During the period of high fat

feeding, 6,581 cc. of mineral acid and 7,493 cc. of base were excreted; that is, an excess of 912 cc. of base for the neutralization of organic acid. The sum of the titratable acid (550 cc.) and the excess of base excreted (912 cc.) is 1,462 cc., which is the amount of organic acid excreted during the 3 days of this period.

R. W. was less susceptible to the change in diet. The excretion of nitrogen, minerals (except calcium), creatine, and titratable acid was increased, but not to the same extent as in the former child. During the normal period 6,033 cc. of acid and 5,970 cc. of base were excreted; that is, 63 cc. of acid in excess of base. The titratable acid of the urine (310 cc.) minus the excess of mineral acid (63 cc.) leaves 247 cc. for the organic acid elimination. During the high fat period 6,681 cc. of acid and 6,806 cc. of base were excreted. The titratable acid of the urine (385 cc.) plus the excess of base over mineral acid excretion (125 cc.) makes 510 cc., which is the organic acid excretion for this period. The values of the plasma carbonates for the high fat periods were in keeping with the urinary findings. Under similar conditions, C. G., the more responsive case, showed a lowering of the carbonate value amounting to 21 volume per cent, whereas R. W.'s plasma carbonates decreased by 12 volume per cent.[8]

The increased elimination of nitrogen during the periods of high fat feeding may possibly be ascribed to an accelerated rate of conversion of protein into carbohydrate. The ammonia in the urine of the more responsive case accounts for but 10 per cent of the excess nitrogen eliminated. The ratio of sulfur to nitrogen lost is about 1: 17 and 1: 21 respectively.

If we assumed that the lost nitrogen was derived from muscle exclusively, then in the case of C. G. about 320 gm. of muscle tissue would have been catabolized. The minerals contained therein would be entirely inadequate to account for the calcium, magnesium, and phosphorus losses. One naturally associates this trio of elements with bone and it is not unlikely that they were derived from this source. The excess of calcium which was excreted during the period of high fat feeding served

[8] The variation in the response of children to acid-producing diets is marked. We have recorded observations concerning this point on a larger series of children in a forthcoming number of the *Am. J. Dis. Child.*

to neutralize more acid (545 cc.) than the surplus of ammonia (480 cc.).

Fitz, Alsberg, and Henderson[9] and others have pointed out the increased elimination of acid phosphates during periods of acidosis. This may account for part of the increased excretion of sodium, potassium, and phosphorus during the period of high fat feeding.

The phosphorus loss of the older child, with the more moderate acid production, was less than one-half that of the other. Calcium was actually retained. The loss of sodium and chlorine, however, was more marked. The diminished excretion of sodium chloride, by the younger child, with the more marked acid production, may possibly be explained by the observation of Fischer[10] that acidosis is associated with a decreased sodium chloride elimination.

The above observations, largely confirmatory in nature, indicate that a considerable loss of minerals, especially *phosphorus* and *calcium*, may accompany short periods of increased acid production in children. The excretion of these elements, in the two cases studied, varies directly with the severity of the acidosis. The pronounced loss of nitrogen during the high fat period is a field for further investigation.

In conclusion, we wish to thank Drs. Dean, Steindler, and Beifeld for their cooperation, and Miss Helen Mougey for the care exercised in the preparation of the diets.

[9] Fitz, R., Alsberg, C. L., and Henderson, L. J., *Am. J. Physiol.*, 1907, xviii, 113.

[10] Fischer, M. H., *J. Am. Med. Assn.*, 1915, lxiv, 325.

LECITHIN. I.

"HYDROLECITHIN" AND ITS BEARING ON THE CONSTITUTION OF CEPHALIN.

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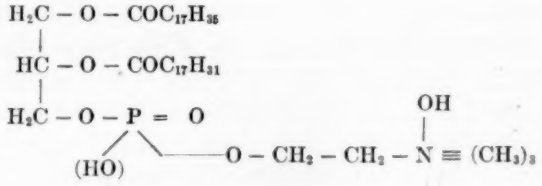
The recent investigations on the chemical structure of lecithin have resulted in many important contributions, all of which point to the correctness of the generally accepted view of its molecular structure,



However, a scrutiny of all the work on lecithin reveals a remarkable incompleteness of each individual investigation. A rigorous proof of the accepted theory requires for lecithin an elementary composition of C = 65.60, H = 10.79, N = 1.74, P = 3.86. It further requires that the nitrogen of the molecule should be composed entirely of choline. Hence, lecithin should not contain even a part of its nitrogen in the form of free amino groups. Still further, it requires a proof of the identity of the fatty acids with those accepted by theory, and finally it requires the isolation of the glycerophosphoric acid.

The work up to the present has satisfied many of the requirements. The fatty acids and the glycerophosphoric acid have been identified; and MacLean has prepared, at least once, a sample of lecithin that was free of amino nitrogen. However, this one sample has been incompletely analyzed. All other samples of lecithin prepared by various workers contained amino

nitrogen in their molecule, and from the standpoint of elementary analysis showed a marked disagreement with the theory. This is well illustrated by the following table.

Author.	Source.	C	H	N	P	Choline.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Thudichum[1].....	Brain.	66.75	18.67	1.81	4.00	—
Baskoff[2].....	Liver.	64.64	10.71	1.95	4.00	—
Heffter[3].....	"	—	—	—	—	25
Stern and Thierfelder[4].....	Egg.	64.63	10.96	1.79	3.95	—
MacLean[5].....	"	64.18	10.60	1.87	3.95	66
Erlandsen[6].....	Heart.	66.29	10.17	1.87	3.95	42
MacLean[7].....	"	66.27	10.32	1.85	3.97	41.4
Eppler[8].....	"	66.46	10.69	1.87	4.03	—
MacLean[7].....	"	—	—	1.89	4.04	68
"	Not given.	—	—	1.85	4.00	66
"	From $CdCl_2$ salt.	—	—	1.87	4.15	98.7

The analytical data obtained by Ritter[9] on hydrolecithin showed better agreement with the theory, and one might be inclined to regard the material of Ritter as such that it contained all the necessary proof in favor of the conventional theory. Unfortunately, Ritter did not determine the amino nitrogen content of the reduced lecithin, and hence failed to furnish definite proof of its purity.

Indeed, the present report contains data unmistakably proving that hydrolecithin of an elementary composition fully harmonizing with the theory may be and generally is impure, containing between 10 and 20 per cent of its nitrogen in the form

[1] Thudichum, J. L. W., The Chemical Constitution of the Brain, London, 1884.

[2] Baskoff, A., *Z. physiol. Chem.*, 1907, lvii, 395.

[3] Heffter, A., *Arch. exp. Path. u. Pharm.*, 1891, xxviii, 100.

[4] Stern, M., and Thierfelder, H., *Z. physiol. Chem.*, 1907, liii, 381.

[5] MacLean, H., *Z. physiol. Chem.*, 1908, lv, 360; 1909, lix, 223; *Biochem. J.*, 1909, iv, 38, 240.

[6] Erlandsen, A., *Z. physiol. Chem.*, 1907, li, 71.

[7] MacLean, *Biochem. J.*, 1915, ix, 364.

[8] Eppler, J., *Z. physiol. Chem.*, 1913, lxxxvii, 241.

[9] Ritter, F., *Ber. chem. Ges.*, 1914, xlvii, 530. Cf. *Reidel's Ber.*, 1913, lvii, 20; 1914, lviii, 15.

of amino nitrogen. Thus the task of the preparation of lecithin having a composition required by the theory and at the same time free of impurities has not yet been accomplished. Efforts in this direction are now in progress in this laboratory.

However, the present finding has a great significance because of its bearing on the structure of cephalin, and the work is presented in its present incomplete state because of this significance. The remarks made earlier in this communication regarding lecithin apply also to cephalin. On the basis of recent work on the hydrolytic products of the substance, a certain structural formula has been assumed. This formula requires an elementary composition of C = 66.17, H = 10.57, N = 1.88, and P = 4.17. However, all samples analyzed, beginning with Thudichum and up to the present by the most recent investigators,[10] consistently had the average composition of C = 60.00, H = 9.30, N = 1.80, and P = 3.80.

On the basis of these considerations, one may argue that if cephalin and lecithin both had the composition required for them by the theory, then a mixture of the two should possess practically the same elementary composition as either one of them in the pure state. On the other hand, if lecithin possessed the composition assumed by the theory and cephalin that found empirically, then a mixture containing 80 per cent of the former and 20 per cent of the latter should possess a carbon content of 64.56 per cent instead of 65.35 per cent. Conversely, if a mixture of the two reduced substances possessed an elementary analysis of C = 65.30, H = 11.20, N = 1.75, P = 3.85, it would justify the conclusion that both lecithin and cephalin possess the composition assumed for them by the theory.

The material analyzed by us contained 80 per cent of lecithin and 20 per cent of an impurity. It was found that the material yielded on hydrolysis besides the choline also the base aminoethanol. Hence it was reasonable to assume that the 20 per cent of impurity consisted of cephalin. If cephalin had the composition found by experience then a substance consisting of 80 per cent of hydrolecithin and 20 per cent of cephalin should have an elementary composition of C = 64.56, H = 10.49, N = 1.75, P =

[10] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1916, xxiv, 41.

3.84. On the other hand, if both lecithin and cephalin possess the structure assigned to them by theory then the above mixture should have the elementary composition found by experiment. Thus the facts presented in this report furnish evidence in favor of the prevailing theory of the molecular structure of lecithin and of cephalin; they also indicate the method by which the pure reduced cephalin may eventually be obtained. Efforts in this direction are now in progress.

In this connection it may be recalled that the product obtained on reduction of cephalin with hydrogen gas in the presence of palladium contained from 62 to 63 per cent of carbon in its molecule, thus approaching nearer than the non-reduced cephalin the theoretical value. The non-reduced cephalin generally obtained is undoubtedly an altered and perhaps oxidized form of the original substance. The nature of the alteration is not known as yet.

EXPERIMENTAL.

Hydrolecithin was first prepared by Paal and Oehme;[11] they reduced an alcoholic solution of egg lecithin with hydrogen and colloidal palladium. The product was obtained as microscopic compact crystals, which sintered at 83–84°, and decomposed over 150° with blackening. Upon hydrolysis the hydrolecithin gave a mixture of stearic, palmitic, and probably myristic acids. The following year Ritter[9] reduced lecithin which had been prepared from fresh, dry egg yolk as follows: The egg yolk was first extracted with petroleum ether and then with ether; the ethereal extract was concentrated and the residue was then extracted with methyl alcohol. The hydrolecithin prepared in this way proved to be distearylhydrolecithin; that is, on hydrolysis it yielded only stearic acid.

Hydrolecithin has been prepared in our laboratory, by Paal's method, from various fractions of egg lecithin, and from lecithin of brain and other organs. During the course of the work it has been noted that lecithin, which has been washed according to MacLean's[7] method, in which the lecithin is ground up with a little water and precipitated with acetone, is reduced more rapidly and more completely than unwashed lecithin. Also, it was

[11] Paal, C., and Oehme, H., *Ber. chem. Ges.*, 1913, xlvi, 1297.

found that the addition of 1 to 2 per cent of acetic acid to the alcoholic solution facilitated the reduction. With fairly concentrated solutions the hydrolecithin separates out during the course of the reduction. This product was brought into solution by warming, filtered from the coagulated palladium, cooled to 0°, and the material which separated out crystallized from dry methyl ethyl ketone until the composition was constant. In most cases it was easily possible to obtain material with correct analytical figures for carbon and hydrogen. In some cases, however, this was impossible, even after repeated crystallization.

Hydrolecithin crystallizes well from methyl ethyl ketone, in which it is insoluble in the cold; it softens between 80 and 90°, turns brown about 100°, starts to melt about 200°, and runs down the tube, giving a dark red liquid, at 235°. The figures vary somewhat, depending upon the rate of heating. The optical activity was determined in chloroform solution and varied between + 5.2° and + 5.4°. The presence of the amino nitrogen-containing body did not seem to affect the value for $[\alpha]_D^{20}$.

$$\text{Sample } 400\ [\alpha]_D^{20} = \frac{9.7822 \times 0.18^\circ}{0.5 \times 0.3254} = +5.4^\circ$$

$$\text{“ } 399\ [\alpha]_D^{20} = \frac{9.2784 \times 0.21^\circ}{0.5 \times 0.3734} = +5.22^\circ$$

$$\text{“ } 429\ [\alpha]_D^{20} = \frac{9.2150 \times 0.24^\circ}{0.5 \times 0.4170} = +5.3^\circ$$

$$\text{“ } 457\ [\alpha]_D^{1} = \frac{9.330 \times 0.23^\circ}{0.5 \times 0.4120} = +5.20^\circ$$

Analysis of these samples gave the following values:

Sample.	C	H	N	P	Ash.	NH_2–N
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
400	65.20	10.89	2.00	4.00	10.61	
399	65.59	11.16	2.37	3.85	10.38	
429	65.60	11.03	1.96	3.79	9.75	7.00
508	65.50	11.30	1.80	3.90	—	20.00
492	—	—	1.98	3.98	9.66	6.00
Theory.	65.37	11.23	1.74	3.84		0

Hydrolysis of Hydrolecithin.

A large quantity (200 gm.) of hydrolecithin was prepared for the purpose of studying its hydrolytic products. This analyzed as follows:

Sample.	C	H	N	P	NH_2–N
508	65.50	11.30	1.80	3.90	20.00

100 gm. of this material were hydrolyzed by boiling with 1 liter of 3 per cent sulfuric acid for 8 hours.[7]

The fatty acid fraction was filtered off and recrystallized from acetone, after boiling about 2 hours with animal charcoal. Again recrystallized from acetone, the acid melted at 69–70°, and on combustion and titration gave figures for *stearic acid.* This confirms Ritter's observation that it is possible to obtain pure distearylhydrolecithin.

The aqueous filtrate was freed from sulfuric acid by the addition of barium hydroxide, concentrated *in vacuo,* precipitated with basic lead acetate, the filtrate freed from lead, and used for the determination of aminoethyl alcohol according to Thierfelder and Schulze.[12] The ethereal extract was concentrated, taken up in water, and the amino nitrogen determined. The theoretical amount of gold chloride was added to the acidified solution. The gold salt separated as long needles after standing 2 days in a desiccator over sulfuric acid. It melts at 184–186°. Trier[13] gives 186–187° for the gold chloride salt of aminoethyl alcohol

[12] Thierfelder, H., and Schulze, O., *Z. physiol. Chem.,* 1916, xcvi, 296.

This method depends upon the fact that calcium oxide does not liberate choline from its hydrochloride, but does free aminoethyl alcohol. The concentrated solution of the mixed hydrochlorides is rubbed up with pure calcium oxide until it is a dry powder and extracted with ether in a Soxhlet apparatus; the flask should contain 0.1 N sulfuric acid to bind the base, otherwise considerable loss occurs. After 27 hours about 96 per cent of the alcohol has been extracted by the ether. The choline may then be extracted with hot alcohol.

[13] Trier, G., *Z. physiol. Chem.,* 1911, lxxiii, 383; 1911–12, lxxvi, 496.

hydrochloride, and Knorr[14] gives about 190° for the synthetic product. It was analyzed by heating to constant weight.

0.2008 gm. substance gave 0.0990 gm. Au.
0.2075 " " " 0.1016 " "

	Calculated:	Found:	
Au	49.17	49.35	48.96

[14] Knorr, L., *Ber. chem. Ges.*, 1897, xxx, 913.

THE COLORIMETRIC DETERMINATION OF HEMOGLOBIN.*

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The method to be described for the determination of hemoglobin depends upon the comparison, in a colorimeter, of carbon monoxide hemoglobin solutions, one of which has a known hemoglobin content. Hoppe-Seyler (1) was the first to describe carbon monoxide hemoglobin and to make use of this stable combination for estimating the hemoglobin content of blood. He devised a "double pipette" for comparing the unknown carbon monoxide hemoglobin solution with the standard carbon monoxide hemoglobin solution, prepared from hemoglobin crystals; but the method never came into general use, because of the many technical difficulties involved. Haldane (2) suggested a much simpler method for comparing carbon monoxide solutions, using the apparatus employed by Gowers (3) for comparing oxyhemoglobin solutions with a picro-carmine standard. This apparatus was later employed by Sahli (4) who prepared an acid hematin standard by adding dilute hydrochloric acid to blood.

A critical discussion of the various methods in use for the estimating of hemoglobin is beyond the scope of this paper. As Haldane (2) has pointed out, artificially colored solutions and tinted glass present great difficulties in standardization with a definite strength of hemoglobin solution. With a certain strength of color solution or tinted glass, it is possible to imitate quite perfectly the tint of a given hemoglobin solution provided the quality of light remains the same. Any variation from these standard conditions, either in quality of light or strength of hemoglobin in solution leads to serious errors. Haldane at-

* A brief report of this paper is given in *Proc. Soc. Exp. Biol. and Med.*, 1916–17, xiv, 175.

tempted to overcome the objection by preparing a carbon monoxide hemoglobin standard (1 per cent solution of a blood having an oxygen capacity of 18.5 per cent) which he considered permanent when kept sealed in a small test-tube in an atmosphere of carbon monoxide. 0.020 cc. of blood, placed in a similar sized and shaped graduated (on scale of 100) test-tube with a small amount of water saturated with carbon monoxide by means of ordinary illuminating gas, are diluted with water, drop by drop, until the unknown and standard tubes mateh in color, whence the percentage of hemoglobin in the unknown may be read off on the graduated scale. In principle, Haldane's method is sound; but certain practical difficulties arise. The standard is not so permanent as was at first thought, and, when water is used, it has been shown by Krogh (5) that the full color of the solution is slow in reaching its maximum. The further criticism is that the method is time-consuming and cumbersome—adding water, drop by drop, and shaking after each addition. Also the color comparison in the two tubes is not sharp. This method has one distinct advantage in that it may be used for the determination of hemoglobin in any species of animal.

Sahli employed Haldane's apparatus and technique; except that he used, as a standard, blood to which dilute hydrochloric acid had been added. There are three serious objections to Sahli's method: first, the standard is not permanent; second, there is considerable delay in the development of the maximum or permanent color, amounting, according to Meyer and Butterfield (6), in some instances to 20 per cent; and third, it cannot be used for the blood of different species.

The spectrophotometer has undoubted accuracy in the hands of skilled operators; but the expense and unavailability together with the difficult technique involved, make it impracticable for general use.

The great variety of methods and apparatus which have been proposed offer eloquent testimony to the unsatisfactory means for the determination of hemoglobin. There is great need for a rapid, accurate, and universally standard method for the estimation of hemoglobin in experimental work and the study of blood diseases in the clinics.

The method which we have found to fulfil the above conditions is as follows:

Method.

Blood is obtained in the usual manner by pricking the finger or lobe of the ear. A 1 per cent solution of blood is made by drawing 0.05 cc. into a special pipette and transferring into 5 cc. of 0.4 per cent ammonia solution—accurately measured with a calibrated pipette or burette into a 12 x 120 mm. test-tube. The blood pipette is rinsed out by drawing into it two or three times the ammonia solution. Ordinary illuminating gas is bubbled rapidly through the ammonia blood solution for 30 seconds, after which, it is compared in a Duboscq colorimeter with a standard carbon monoxide hemoglobin solution set at 10. The average of at least four readings is taken. The calculation is simple, $\frac{10}{R} \times 100 =$ per cent hemoglobin.

Manner of Obtaining Blood.—With sufficient care the usual clinical method for obtaining small amounts of blood by pricking the ear or finger is satisfactory. A free flow is essential. Any undue manipulation or squeezing of the part should be avoided because an error of 5 or 10 per cent may be introduced by diluting the blood with tissue juice. Where there is marked anemia requiring larger amounts of blood than 0.05 cc., or where there is difficulty in obtaining blood from the ear or finger, venous puncture should be used, coagulation being prevented with oxalate or citrate salts. It is often practical and convenient to combine the determinations of hemoglobin with other blood analyses, where venous puncture is required. If blood has been drawn by venous puncture care must be taken that the corpuscles and serum are well mixed before filling the pipette. The blood should never be shaken violently before measuring, because it becomes filled with air bubbles. The mixture of corpuscles and serum may best be accomplished by first giving the receptacle a circular motion and finally stirring briskly with a glass rod or measuring pipette which is filled while stirring the blood.

Pipette for Measuring Blood.—The pipettes are made of millimeter glass tubing calibrated to contain 0.05 cc. and 0.10 cc. The pipettes are easily made in any laboratory from straight tubing, and require no blowing, the point being rounded off on an emery wheel. In this way time and expense are saved, since pipettes

obtained from glass blowers require recalibration before use. It has been found that water may be used for this calibration, as pipettes which have been calibrated with both mercury and water check sufficiently well. The advantage of having the pipette calibrated to contain 0.10 cc. as well as 0.05 cc. is obvious. In bloods with a low hemoglobin content, 0.05 cc. may not be sufficient to give the color necessary for accurate color comparison in the colorimeter. A pipette of this type, and used in the manner described, is capable of measuring 0.05 cc. of blood with an accuracy of 0.2 per cent.

Ammonia Solution.—Ammonia solutions, containing 4 cc. of strong ammonia in 1 liter of water, suggested by Krogh (5) are used, because the full color of the carbon monoxide hemoglobin develops at once.

Saturation with Carbon Monoxide.—Ordinary illuminating gas as a source of carbon monoxide has proven entirely satisfactory. It was thought that there might be substances other than carbon monoxide in the gas which might form hemoglobin compounds and interfere with the determination. Accordingly, pure carbon monoxide was prepared by heating oxalic acid with concentrated sulfuric acid and passing the gas produced through sodium hydroxide to free it from carbon dioxide. As far as could be determined on comparison of the two solutions in the colorimeter the colors were identical. Oxyhemoglobin solutions are very unstable. Hence it is necessary, after transferring the blood to the ammonia solution, to saturate with carbon monoxide within an hour. After saturation with carbon monoxide, the solution may, on carefully stoppering and protecting from light, be placed in the ice box and the determination made at leisure. Saturation of the blood should be carried out under a hood. If the laboratory does not possess a hood, the saturation may be accomplished under a funnel, attached to a small water vacuum pump, to remove the gas.

Standard Hemoglobin Solution.—Haldane's standard of a 1 per cent solution of a blood having an oxygen capacity of 18.5 per cent is used. It has been shown by Haldane and Smith (7), Butterfield (8), Barcroft (9), and others, that the oxygen capacity of the blood depends upon its hemoglobin content. A blood of 18.5 per cent oxygen capacity contains approximately 14 gm.

hemoglobin per 100 cc. Although the blood of normal men in this country, as shown by Meyer and Butterfield (6), Williamson (10), and also as we have found by use of the method here described, contains on the average 16.6 gm. of hemoglobin, which would correspond to an oxygen capacity of about 22 per cent, it was thought best to keep Haldane's standard for the present. It is a simple matter to compute the gm. of hemoglobin in any given blood from the results obtained.

The standard hemoglobin solution is prepared as follows: A quantity of defibrinated human or ox blood is obtained. The oxygen capacity is determined by the method of Van Slyke (11). The blood may also be standardized by a spectrophotometer or solutions made from hemoglobin crystals prepared in the manner described by Butterfield (8). We have checked several times our standard and found that the oxygen capacity method for standardization is most convenient and satisfactory. The blood is diluted with 0.4 per cent ammonia solution so as to make a 20 per cent solution of a blood with an oxygen capacity of 18.5 per cent. This 20 per cent blood solution is then saturated with carbon monoxide by bubbling through it illuminating gas for 10 minutes. A drop of caprylic alcohol prevents troublesome foaming. The glass tube through which the gas is passed into the blood solution is withdrawn slowly and the bottle stoppered immediately. Rubber corks must not be used in connection with hemoglobin solutions. The cork should be sealed in with paraffin and the solution, protected from light, kept in the ice chest. Such a solution thus protected will keep for months. Several solutions now nearly a year old prepared in this manner have shown no deterioration. 5 cc. of this 20 per cent blood solution made up to 100 cc. with 0.4 cc. of ammonia solution and saturated with carbon monoxide, make the 1 per cent standard for use in the colorimeter and may be prepared from time to time as desired. The 1 per cent standard for routine use may be kept in a dark glass or black painted aspirator bottle, the lower opening of which is provided with a cork, through which passes a glass tube with a ground glass cock for withdrawing small amounts of solution. A glass tube is put through the cork in the top of the bottle and extends to the bottom. Both corks should be sealed with paraffin. This glass tube is connected with an open

gas fixture in order that when solution is withdrawn from the bottom, gas rather than air will enter to replace it. Solutions thus prepared may keep for several weeks; but, as a precaution, it is advisable to make fresh 1 per cent solutions frequently; *i.e.*, every 2 or 3 weeks. It should be remembered that dilute hemoglobin solutions are less stable than concentrated solutions; and that hemoglobin solutions keep best in the cold and protected from light. The first indication of solution deterioration is a change in color from the characteristic cherry-red of carbon monoxide hemoglobin to a red with a brownish tinge, due to the formation of methemoglobin.

Comparison in Colorimeters.—The Dubosecq or Kober colorimeters have proven to be by far the most accurate and satisfactory instruments for this colorimetric work. Other colorimeters, however, may be used. The difficulties encountered are those inherent in all colorimetric work and in this connection reference to Kober's (12) article may be made. The color of the carbon monoxide hemoglobin, because of the relatively low stimulus threshold for the eye, is admirably suited to colorimeter comparison, slight differences being easily detected. We prefer to use the daylight from a north window. Satisfactory results are, however, obtainable with artificial light when "daylight glass" is used between the source of light and the solution. Considerable experimentation with light filters has failed to improve on the accuracy with which the comparison may be made. No difficulty should be experienced in making the readings check within 0.2 of a single division on the colorimeter scale.

Color comparisons are most accurate when the unknown hemoglobin solution reading falls between 9 and 11 on the colorimeter scale. If the reading of the unknown falls below 8 or above 12, another sample should be taken and the dilution made such that the reading will fall within these limits. This is easily accomplished by varying the amounts of blood and ammonia solution, making the necessary correction in the colorimeter. 2 cc. of solution is adequate for the Kober instrument and 5 cc. for the Dubosecq. If a Dubosecq or a Kober colorimeter is not available, the Hellige instrument may be used. The 1 per cent hemoglobin standard may be sealed with paraffin into the wedge and the wedge, when not in use, kept in the ice box

and protected from light. Attention should be called to the fact that the scales of the Hellige colorimeter are often inaccurately placed. The standard solution must be checked against itself and the scale adjusted so as to read 100 when the color in the cup and wedge match. We have found more difficulty in obtaining accurate checks with this instrument than with the Duboscq or Kober colorimeters; but with care the error should not exceed 2 per cent. 2 cc. of solution is sufficient for the Hellige cup, hence the Sahli pipette, which contains 0.020 cc. of blood, may be used with this colorimeter. As large errors have been found in the calibration of the Sahli pipettes, it is necessary to recalibrate them before using.

Accuracy of the Method.—The accuracy of the method depends to a large extent on the care of the operator in carrying out the various details of the technique. The several steps involved in the method are common chemical procedures with known limits of accuracy. With care duplicate determinations are close and the error should not be more than 1 per cent. A series of ten determinations was made on the same blood. In eight of the ten, the first reading of the colorimeter was in every case exactly the same. In the two remaining a difference of 0.1 of a division on the colorimeter scale occurred.

In the following table are presented a few determinations by the method described using the Duboscq and Hellige colorimeters and comparing with the values estimated from the oxygen

Blood sample.	Hemoglobin determinations.			Difference between Duboscq and oxygen capacity.	Percentage difference.
	Duboscq.	Hellige.	Oxygen capacity.		
	per cent	*per cent*	*per cent*		
1	105.5	104.8	106.5	+1.0	0.94
2	95.2	92.0	94.2	−1.0	1.06
3	128.3	125.0	129.5	+1.2	0.93
4	112.5	115.2	111.7	−0.8	0.68
5	116.2	116.3	116.4	+0.2	0.17
6	79.4	84.0	80.5	+1.1	1.36
7	119.2	115.0	119.4	+0.2	0.17
8	84.0	85.0	83.2	−0.8	0.95
9	82.0	82.0	82.6	+0.6	0.73
10	108.6	—	108.1	−0.5	0.46

capacity of the blood. The first ten blood samples of a large series were chosen and illustrate the error in estimation which may be expected. Except in blood Sample 6, no variation between the colorimetric determination (Duboscq) and the oxygen capacity method is greater than 1.0 per cent.

Advantages.—(1) Single determinations may be carried through in 2 minutes. (2) An accuracy within 1 per cent is easily obtained. (3) The standard solution is easily and adequately controlled. (4) Similar solutions are used for comparison, making the color fields, within the limits of the colorimeter, identical. (5) The apparatus required is found in any well equipped laboratory.

SUMMARY.

A method for the determination of hemoglobin colorimetrically with an accuracy of 1 per cent is described.

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GASOMETRIC DETERMINATION OF THE OXYGEN AND HEMOGLOBIN OF BLOOD.

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The apparatus described in a previous paper[1] for determining the carbonic acid content of plasma may be used with equal facility for determining the oxygen content and the oxygen-binding capacity (hemoglobin) of blood.

For determination of the *oxygen capacity* as a measure of the hemoglobin 3 or more cc. of blood are introduced into a separatory funnel or bottle and distributed in a thin layer about the inner wall, so that maximum contact with the air and complete saturation of the hemoglobin with oxygen are assured. The vessel is rotated for a few minutes so that the blood is kept in a thin layer, or it may be shaken for 15 minutes or longer on a mechanical shaker. The saturated blood is transferred to a heavy test-tube or cylinder.

The blood gas apparatus is now prepared by introducing into it five drops of redistilled caprylic alcohol and 6 cc. of ammonia solution made by diluting 4 cc. of concentrated ammonia to a liter. If saponin powder is available, as much is added to the 6 cc. of ammonia while in the cup of the apparatus as will stick to the end of a glass rod. After the ammonia has been introduced into the 50 cc. chamber of the apparatus the latter is evacuated in the manner described in the previous article, and the air is extracted from the ammonia solution by shaking for about 15 seconds. The extracted air is expelled, and the extraction completed to make sure that no air is left in the solution. Finally, about 2 cc. of the air-free ammonia are forced up into the cup of the apparatus.

The aerated blood is now thoroughly stirred with a rod to

[1] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

assure even distribution of the corpuscles, and a sample is drawn into a 2 cc. pipette and run under the ammonia in the cup of the apparatus. (The lower delivery mark of the pipette should be 3 or 4 cm. above the tip. A pipette calibrated for complete delivery would be inconvenient for placing the entire sample of blood below the layer of ammonia.) The blood is now run from the cup into the 50 cc. chamber, the ammonia layer following the blood and washing it in. A few additional drops of ammonia may if necessary be added from a dropper to make the washing complete.

The blood and ammonia in the chamber are mixed and allowed to stand until the blood is *completely laked.* This requires about 30 seconds when saponin is present, 5 minutes when it is not. After laking is complete 0.4 cc. of a saturated potassium ferricyanide solution is introduced to set free the oxygen combined with the hemoglobin. (The cyanide solution is made air-free by boiling or by shaking in an evacuated flask and is kept in a burette under a layer of paraffin oil 2 or 3 cm. thick to exclude air.) The apparatus is now evacuated by lowering the levelling bulb until only a few drops of mercury remain above the lower stopcock, and is shaken, preferably with a rotary motion, to whirl the blood in a thin layer around the wall of the chamber. If the blood was completely laked before the cyanide was added, extraction of the oxygen may be completed by half a minute of efficient shaking. The extracted solution may be drawn into the bulb of the apparatus below the lower cock and the extracted gas measured over mercury as in the determination of carbon dioxide.[2] Or, since the water does not absorb oxygen rapidly enough to cause an error, the solution may be left in the 50 cc. chamber during the reading of the gas volume, the levelling bulb being held at a sufficient height to balance the column of water solution.[2] Finally, in order to make certain that all the oxygen was obtained by the first extraction, the apparatus is evacuated once more and the blood shaken again for a half minute. If the reading shows no increase, it is evidence that all the oxygen was extracted by the first evacuation. If there is an increase the extraction must be repeated again. If the blood is completely

[2] Van Slyke, *J. Biol. Chem.*, 1917, xxx, 353.

laked before addition of the ferricyanide, the first shaking practically always removes all the oxygen from the blood solution. Even when laking has been incomplete, however, or the first extraction of the blood otherwise made incomplete, the determination is not lost; for the right result will be obtained if the extraction is repeated until the reading becomes constant.

After each analysis it is well to wash out the 50 cc. chamber of the apparatus with the dilute ammonia solution, as a black precipitate is formed by reaction of the reagents with the mercury. Unless this precipitate is removed it tends to coagulate after a few analyses and interfere with further determinations.

After the blood has been saturated with air the entire procedure above outlined, including the final cleaning of the apparatus, is done in routine determinations in 7 or 8 minutes.

In order to calculate the oxygen bound by the hemoglobin it is necessary to subtract from the gas measured the volume of air physically dissolved by the 2 cc. of blood at atmospheric pressure and the prevailing room temperature. The volume of gas thus corrected may be reduced to standard conditions by multiplying by $(0.999 - 0.0046\ t) \times \frac{\text{barometer}}{760}$, t being the temperature in degrees centigrade. The volumes of air dissolved by the blood at different temperatures are given in Table I. They are calculated in accordance with Bohr's finding, that the solubility of gases in average whole blood is 90 per cent of their solubility in water. The table also gives factors by which one may transpose the readings directly into terms of volume per cent of chemically bound oxygen in the blood, or of per cent hemoglobin on the basis of Haldane's normal average, 18.5 per cent of oxygen in the blood being taken as equivalent to 100 per cent hemoglobin.[3]

Unless one is well experienced with the conditions used for saturating the blood with oxygen, it is advisable, after one portion of a blood sample has been analyzed, to aerate the remainder a second time and repeat the determination, in order to make certain that the hemoglobin of the first portion was completely saturated with oxygen.

[3] Haldane, J., and Smith, J. L., *J. Physiol.*, 1899–1900, xxv, 331.

TABLE I.

Factors for Calculating Results from Analysis of 2 Cc. of Blood Saturated with Air.

Temperature.	Air physically dissolved by 2 cc. of blood. Subtract from gas volume read in order to obtain corrected gas volume, representing O_2 set free from hemoglobin.	Factor by which corrected gas volume is multiplied in order to give:	
		Oxygen chemically bound by 100 cc. of blood.	Per cent hemoglobin, calculated on the basis of 18.5 per cent oxygen = 100 per cent hemoglobin.
C.°	cc.	cc.	per cent
15	0.037	$46.5 \times \frac{B}{760}$	$251 \times \frac{B}{760}$
16	0.036	46.3 "	250 "
17	0.036	46.0 "	249 "
18	0.035	45.8 "	247 "
19	0.035	45.6 "	246 "
20	0.034	45.4 "	245 "
21	0.033	45.1 "	244 "
22	0.033	44.9 "	242 "
23	0.032	44.7 "	241 "
24	0.032	44.4 "	240 "
25	0.031	44.2 "	239 "
26	0.030	44.0 "	237 "
27	0.030	43.7 "	236 "
28	0.029	43.5 "	235 "
29	0.029	43.3 "	234 "
30	0.028	43.1 "	233 "

The following example illustrates the calculation:

Observed gas volume, at 20°, 750 mm..... 0.450 cc.
Correction for dissolved air..... 0.034 "
Corrected gas volume..... 0.416 "
$0.416 \times 44.8 = 18.65$ volume per cent oxygen.
$0.416 \times 243 = 101$ per cent hemoglobin.

While the above description and the table are prepared to fit the analysis of 2 cc. samples of blood, which seems the desirable amount for ordinary purposes, either more or less may be taken, the volumes of dilute ammonia and ferricyanide used being changed proportionally. The following data show that good results are obtained with as much as 3 or as little as 1 cc. of blood,

although the error must be greater in the latter case because of the small volumes of gas obtained for the final reading.

TABLE II.

Determinations of the Oxygen Capacity of Ox Blood, Using 1 to 3 Cc. Samples.

Volume of blood sample.	Temperature.	Barometer.	Gas volume obtained.	Gas volume corrected for dissolved air.	Volume of O_2 reduced to 0°, 760 mm. bound by 100 cc. of blood.	Per cent hemoglobin, calculated on basis of 18.5 per cent O_2 = 100 per cent hemoglobin.
cc.	*°C.*	*mm.*	*cc.*	*cc.*	*cc.*	*per cent*
3.00	20	752	0.835	0.784	23.45	126.7
2.00	20	752	0.558	0.524	23.49	126.9
1.00	20	752	0.281	0.264	23.67	127.9

For determination of the *oxygen content* of the venous blood as drawn from the body, the preliminary saturation with air is omitted, and precautions, such as are described by Lundsgaard in the accompanying paper,[4] are observed in order to prevent contact of the sample with air. From the point where the blood sample is transferred to the cup of the apparatus for analysis the technique is identical with that described above.

The calculation of the oxygen content is somewhat different from that of the oxygen capacity. The physically dissolved oxygen of venous blood is negligible, and the nitrogen as determined by Bohr is 0.9 volume per cent, reduced to 0°, 760 mm.[5] Therefore the volume of gas obtained in the apparatus is at once multiplied by the factor $(0.999 - 0.0046\ t) \times \frac{\text{barometer}}{760}$ to reduce it to standard conditions. In case 2 cc. of blood have been used, the values of this factor in Column 3 of Table I may be used; in this case they give the content of the blood in chemically bound oxygen plus the dissolved nitrogen gas. The result in cc. of gas per 100 cc. of blood is diminished by 0.9 cc. in order to correct for that amount of nitrogen gas present.

The nature of the results obtained with the method and the degree of accuracy indicated by the agreement of duplicates are shown by Lundsgaard's figures,[4] while Palmer's show the agree-

[4] Lundsgaard, C., *J. Biol. Chem.*, 1918, xxxiii, 133.

[5] Bohr, C., in Nagel, W., Handb. Physiol. Menschen, 1909, i, 117.

ment of the gasometric with the colorimetric determination of hemoglobin[6] by the most accurate use of the colorimetric technique. It consequently appears unnecessary to reproduce in this paper further data to illustrate the results obtained with the method.

SUMMARY.

The apparatus previously described for determination of carbon dioxide in blood is used with a similar technique for determination of oxygen. The oxygen is set free from combination with hemoglobin within the apparatus by addition of ferricyanide, is extracted in a Toricellian vacuum, and measured at atmospheric pressure, a few minutes sufficing for an accurate determination.

[6] Palmer, W. W., *J. Biol. Chem.*, 1918, xxxiii, 119.

STUDIES OF OXYGEN IN THE VENOUS BLOOD.

I. TECHNIQUE AND RESULTS ON NORMAL INDIVIDUALS.

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The term oxygen content of the venous blood has been used in the literature in two senses. It is applied to the amount of oxygen in the blood in the right heart and also the amount in the venous blood of some organ or group of organs.

In the blood of the right side of the heart the oxygen can be determined by using the lungs plus a bag (1–5) or a part of the lungs (6) as a tonometer. The air in the lungs is brought to equilibrium with the blood gas and a sample of the air is analyzed. In animals, samples of blood can be drawn through a cannula introduced into the heart.

Samples of venous blood from single organs can be obtained in animals when the technical difficulties can be overcome. Extensive studies have been made in the last few years, particularly by Barcroft and his associates (7).[1]

In adults the superficial veins of the limbs and neck, particularly of the arm (vena mediana) are the only sources from which venous blood can be obtained.[2] That means that only blood coming from a limited region, consisting chiefly of muscles, can be studied. Consequently the field of investigation is limited. The conditions can be varied voluntarily only to a small extent. The results have to be interpreted chiefly in an empirical way and by means of general clinical observation. In spite, however, of limitations and *a priori* theoretical difficulties in interpreting

[1] For bibliography see Barcroft (7).

[2] In the Pediatric Department of Johns Hopkins Hospital venous blood is obtained by puncturing the sinus sagittalis in babies. This method makes it possible to obtain venous blood without stasis from children where the superficial veins are too small.

the results, the question of the amount of oxygen in the venous blood in health and disease has always been considered important by physiologists and clinicians, although few investigations have been made. Not only are the observations on pathological cases few, but the determinations on normal individuals are even more scarce. The reason for this is probably that we did not have any means of determining blood gases in small quantities of blood until the Haldane-Barcroft method was devised.

Kraus (8) determined the oxygen and CO_2 in the venous blood of a series of normal and pathological cases. Using the old blood-gas pump it was necessary for him to draw large quantities of blood, a circumstance which prevented repeated determinations in the same person. He found in some instances of incompensated heart cases very low values for the venous oxygen compared with that of normal individuals. Using the Haldane-Barcroft method, Morawitz and Röhmer (9) did eighteen determinations on three normal people, twenty determinations on nineteen patients with anemia, and one determination on a patient with polycythemia. In some instances they found an increased consumption,[3] in other instances a normal consumption in their patients. Means and Newburgh (10) using the Haldane-Barcroft method found, like Kraus, low values in four patients with incompensated heart diseases compared with those found in three normal subjects.

We know that in normal individuals the oxygen content of the venous blood is about 13.5 volume per cent, or two-thirds of the total amount in the blood when it is saturated. The values for the oxygen in the blood from an arm vein have not differed materially from the values obtained from the average blood flowing to the right side of the heart. A similar value has been obtained by calculation in the experiments on the blood flow done by Krogh and Lindhard's (11) method.[4] In animals very varying figures have been obtained in blood from different organs (Barcroft, 7).

However, our knowledge has been only approximate. Great variations occur; how great, or how caused, we do not know. It is therefore necessary to establish the limits in not too small a

[3] The term "consumption" means the percentage of oxygen absorbed in the circulation. The arterial blood was considered saturated with oxygen.

[4] See discussion of blood flow experiments in Paper II, *J. Exp. Med.*, 1918, xxvii, in press.

number of normal individuals before we can hope to be able to interpret results from abnormal individuals. This paper is a report of thirty-eight determinations on twenty normal individuals.

Technique.

Two operations are involved: first, the drawing of a sample of venous blood; and second, the determination of its oxygen content. The determination of the oxygen has been done by Van Slyke's method (12). This method is quicker, easier to handle, and more exact than the Haldane-Barcroft, where the great volume of foreign air in the apparatus requires elaborate facilities for keeping the temperature constant. A determination with Van Slyke's apparatus (including cleaning of the machine) requires 5 to 10 minutes. The figure can be read with an accuracy of 0.004 cc. Adding to this the inevitable error in measuring and delivering 2 cc. with a pipette, we can say that the difference between duplicates can always be kept within 0.01 cc. of oxygen, which is 0.5 volume per cent of the 2 cc. of blood analyzed. As will be seen from the tables, duplicates usually agree much more closely. Greater differences are caused only by gross errors, such as by imperfect mixture of the corpuscles in the blood, or by allowing time for a slow oxidation (or deoxidation) of the blood between the two determinations (see below).

The other part of the technique, the obtaining of a sample of blood, has been as follows: The arm from which the blood is drawn rests comfortably at the side of the body on a small, moderately soft pillow. The blood is drawn without stasis and the pulse and respiration are counted from the moment the blood appears.[5] The apparatus for blood drawing consists of a very sharp needle connected by means of a 3 to 4 cm. rubber tube to a glass pipette of about 0.5 cm. bore. The pipette and the rubber have a film of sodium oxalate crystals in them. This is obtained by wetting the interior with a saturated solution of oxalate, which is dried with a current of air. In drawing the blood one puts the upper end of the pipette into the mouth (some

[5] It might be more logical to start counting the pulse a half minute before the blood appears. However, the pulse rate usually keeps almost constant under the procedure if it is skilfully done.

cotton wool will prevent saliva from flowing down) and quickly introduces the needle in the proximal direction. After a little practice it is generally possible to obtain blood at the first puncture. If the needle is dull, unnecessary pain will be caused and the vein will probably escape the needle. If the point is too long, it will almost invariably hit the opposite wall of the vein before the point is entirely within. The needle is introduced best at an angle of about 50° with the surface of the arm. The opening of the needle must be kept downward or to the side so that the upper wall of the vein will not close the opening when the blood is being drawn. In drawing the blood only a gentle suction is necessary, and if air should happen to be sucked through the blood the sample must be thrown away and a new pipette used. The needle and rubber can often be kept *in situ* while another pipette is connected with the rubber. When a sufficient amount of blood (6 to 12 cc.) has been drawn, the upper end of the pipette is closed by a finger and the needle withdrawn. Then the needle and the rubber are taken away from the pipette and the blood is discharged into a cylinder (2 cm. wide and with a few oxalate crystals in the bottom) below a layer of white mineral oil at least 2 cm. deep. Just before the last 0.5 to 1 cc. of blood has run out, the pipette is again closed with a finger and withdrawn, because the upper part of the blood in the glass pipette has been oxidized and must be discarded. The blood in the cylinder is then stirred up with a glass rod to secure a good mixing with the oxalate. From the cylinder 2 cc. samples of blood are introduced into Van Slyke's apparatus below a layer of evacuated dilute ammonia as described by Van Slyke (12). Before a sample is taken the blood must be stirred up carefully to secure homogeneity. If the blood has been kept in an ice box for some time (see below) it is often difficult to secure homogeneity, even after it has gained room temperature. It gets more sticky and adheres to the sides of the cylinder. It is therefore advisable to do the determinations on fresh blood. After the blood has been stirred with a rod, a 2 cc. pipette is introduced, the upper end being closed to prevent mineral oil from coming up, and after a few seconds' additional stirring with the pipette, the sample is sucked up and discharged into the apparatus. The pipette must be calibrated for 2 cc. outflow, and may be calibrated either for complete discharge or for discharge to a mark

on the lower stem. The latter is preferable, but the pipette for complete discharge gives in practice equally good results, because only a very little surface of blood touches the air, so the whole blood column may be used without introducing any appreciable error. The calculation of the amount of oxygen in the sample is simple (see p. 131, Van Slyke's paper (12)). The determination of the total oxygen-combining power of the blood has in some instances been done with the Van Slyke apparatus, in other instances with Palmer's colorimetric method (13). If the blood has been kept in an ice box for some time, it may be impossible to rely upon the homogeneity. Consequently when the main sample of blood is placed under an oil layer, a portion of about 1 or 2 cc. is transferred to a special dish without oil and used for the Palmer determination. It is advisable to have a glass bead in this dish to stir the sample.

RESULTS.

The results are given in Tables I and II. Besides the date and hour of bleeding and of the determination, the pulse and respiration during the bleeding, three groups of figures are given. (1) The oxygen content of the venous blood in volume per cent corrected for temperature and pressure. In all instances double determinations have been done and the average has been taken. (2) The oxygen-combining power of the blood (hemoglobin), in most instances calculated from the determination of the hemoglobin by Palmer's method.[6] (3) The difference between the oxygen in the venous blood and the total oxygen-combining power of the hemoglobin.

This last figure is important. It is difficult to attach much significance to the figure for the venous oxygen, unless we know what percentage of the total oxygen-combining power of the hemoglobin the venous oxygen represents. The venous oxygen uncontrolled by hemoglobin determinations is as incomplete as a nitrogen excretion considered without relationship to the nitrogen intake. We have termed the difference between the venous oxygen and the total oxygen-combining power of the hemoglobin

[6] I am greatly indebted to Dr. Walter W. Palmer, who has done the majority of these determinations.

the *oxygen unsaturation* of the venous blood, since it represents that portion of the hemoglobin which does not have its oxygen-binding values saturated. The term unsaturation is used in a sense analogous to that in which it is used in organic chemistry, defined by Webster as "falling short of saturation, not combined to the greatest possible extent."[7]

Thirty-eight determinations on twelve different normal individuals have been done. In Table I are given twenty determinations on one person, and in Table II eighteen determinations on eleven different people. Care has been taken to draw the blood from a person resting as completely as possible, because we know that an increase in the metabolism necessarily is followed by an increased oxygen consumption. Before the drawing of the blood the subjects rested on a couch for some time, in some experiments 10 minutes, in others half an hour. In some instances the blood was taken in the morning, before getting up. It does not seem to make any difference whether the resting period is 10 minutes or 30. The oxygen content of the blood taken in the morning had a tendency to be low, particularly when the person had been awake only a few seconds. The average figure for the oxygen content in the twenty determinations on No. 1 is 13.7 volume per cent, with a maximum of 16.84 volume per cent and a minimum of 9.55. The average figure for the eighteen determinations on the other cases is 13.6 volume per cent, with a maximum of 17.98 and a minimum of 10.36 volume per cent.

The total oxygen-combining power (the hemoglobin) has varied from 21.44 (hemoglobin 116 per cent) to 17.50 (hemoglobin 94.5 per cent). This must, of course, influence the amount of oxygen in the venous blood. In the determination in No. 27, for instance, the amount of oxygen in the venous blood is greater than the total capacity of the hemoglobin in No. 36. Such measures illustrate the necessity of taking as a measure of oxygen

[7] The term oxygen consumption has been applied to the figure for which we prefer the more exact term unsaturation. The use of consumption in this connection implies that all the venous oxygen which the venous blood requires in order to be saturated is lacking for the reason that it has been consumed by the tissues. This assumption would be correct if one could always be certain that the arterial hemoglobin is 100 per cent saturated with oxygen. Since one cannot be certain of this, it seems preferable to use the term unsaturation, which is free from the above assumption.

Determinations in Venous Arm Blood of Oxygen, Hemoglobin, and Oxygen Unsaturation in a Normal Subject.

Determination.	Bleeding.			Oxygen content of venous blood.					Hemoglobin (Palmer method).	Calculated oxygen capacity.	Difference between oxygen capacity and oxygen in venous blood = oxygen unsaturation.	Pulse.	Respirations.	Remarks.
				Sample 1.		Sample 2.		Average.						
	Date.	Hour.	Arm.	Hour.	Vol. per cent.	Hour.	Vol. per cent.							
	1917								*per cent*	*vol. per cent*	*vol. per cent*			
1	Jan. 25	3.30	Right.	4.20	14.20	4.50	14.22 14.24	14.22	108	19.96	5.75	78	14	10 min. rest.
2	" 26	3.20	"	3.25	13.95	5.25	14.20 14.62	14.26	—	(19.96)	5.83	74	12	10 " "
3	" 27	4.30	"	4.35	14.50	5.10	14.50	14.50	—	(19.96)	5.42	86	20	10 " "
4	" 29	12.10	Left.	12.30	13.42	12.50	13.42	13.42	—	(19.96)	6.54	70	16	10 " "
5	" 30	4.30	"	4.40	15.40	4.50	15.20	15.30	108	19.96*	4.66	90	14	10 " "
6	" 31	12.20	Right.	12.40	15.80	3.15	15.75	15.78	—	(19.96)	4.18	78	14	10 " "
7	Feb. 6	10.40	"	10.50	16.80	11.10	16.88	16.84	108	19.96	3.12	80	14	10 " "
8	" 6	12.10	"	12.20	12.90	12.35	13.22	13.06	—	(19.96)	6.90	70	14	10 " "
9	" 13	5.10	"	5.15	13.64	5.30	13.64	13.64	110	20.34	6.70	71	14	10 " "
10	" 28	12.20	Left.	12.30	14.25	12.50	14.28	14.27	—	(18.83)	4.56	73	11	½ hr. rest.
11	Mar. 1	11.30	"	11.55	16.15	12.20	16.11	16.13	—	18.83*	2.70	78	12	½ " "
12	Apr. 4	11.00	Right.	12.10	15.06	12.30	14.35	14.70	100	18.50	3.74	72	14	½ " "
13	May 30	7.15	"	7.35	9.54	7.55	9.56	9.55	—	(18.50)	8.95	—	—	Before getting up. Just awakened.
14	" 31	7.15	"	7.30	15.02	7.45	14.99	15.01	100	18.50	3.49	76	14	Before getting up. Had been awake for ½ hour.
15	June 1	7.15	"	7.50	13.97	8.30	14.01	13.99	—	(18.50)	4.51	80	16	Awake for ½ hr.
16	" 2	7.15	"	7.30	12.34	7.45	12.37	12.36	—	(18.50)	6.14	72	14	" " ½ "
17	" 3	7.30	"	7.45	9.79	8.10	10.74	10.27	100	18.50	8.23	66	12	Deep sleep just before blood was taken.
18	" 4	7.15	Left.	7.30	12.54	11.00	12.49	12.52	—	(18.50)	5.98	70	12	Before getting up. Had been awake for ½ hr.
19	" 6	7.15	"	10.15	12.24	11.45	12.20	12.22	102	18.87	6.65	68	12	Awake for ½ hr.
20	" 7	7.15	Right.	7.30	12.23	7.45	11.97	12.10	102	18.87	6.77	70	14	" " ½ "

* Determination of the total oxygen-combining power of the blood done by the Van Slyke apparatus.

TABLE II.

Determinations in Venous Arm Blood of Oxygen, Hemoglobin, and Oxygen Unsaturation in Eleven Normal Subjects.

Determination.	Bleeding.			Oxygen content of venous blood.					Hemoglobin (Palmer method).	Calculated total oxygen capacity.	Difference between oxygen capacity and oxygen in venous blood = oxygen unsaturation.	Pulse.	Respirations.	Remarks.	Name.
				Sample 1.		Sample 2.		Average.							
	Date.	Hour.	Arm.	Hour.	Vol. per cent.	Hour.	Vol. per cent.								
	1917								*per cent*	*vol. per cent*	*vol. per cent*				
							15.20								
21	Feb. 1	12.10	Right.	12.20	15.50	12.40	15.42	15.37	115	21.26	5.89	70	14	10 min rest.	
22	" 7	11.40	Left.	11.50	16.03	12.10	16.24	16.14	116	21.44	5.30	84	13	10 " "	Dr. St.
23	" 7	12.40	"	12.50	16.76	1.10	16.66	16.71	116	21.44	4.73	81	14	10 " "	
24	Feb. 8	12.15	Left.	12.25	11.66	3.00	11.98	11.82	—	17.48*	5.66	55	12	10 min. rest.	
25	Apr. 9	12.00	"	3.20	12.27	3.30	12.25	12.26	99	18.48	6.22	68	—	10 " "	Dr. F.
26	" 30	12.00	Right.	12.30	13.17	1.00	13.04	13.11	100	18.50	5.39	58	19	½ hr. rest.	
27	Feb. 5	11.20	Left.	11.40	18.01	11.55	17.95	17.98	113	21.11*	3.13	68	14	10 min. rest.	
28	" 5	12.40	"	12.50	15.82	1.10	16.25	16.04	—	—	5.07	—	—	10 " "	Dr. V. S.
							14.04								
29	Feb. 9	10.00	Right.	10.10	13.93	10.25	14.15	14.04	101	18.86	4.82	78	14	10 min. rest.	
30	" 9	1.15	"	1.30	12.35	2.10	12.61	12.48	—	18.86	6.38	76	14	10 " "	Dr. Bl.

31	June 3	11.15	Left.	11.30	12.00	12.15	12.00	12.00	108	20.00	8.00	54	22	½ hr. rest.	
32	" 14	3.30	"	3.50	10.62	4.10	10.68	10.65	98.4	18.30	7.65	52	20	½ " "	Dr. L.
33	Feb. 2	10.45	Right.	10.55	13.15	11.10	13.10	13.13	107	19.80*	6.67	70	18	10 min. rest.	T
34	Feb. 1	4.20	Left.	4.30	15.55	4.45	15.45	15.50	100	18.50	3.00	88	18	10 min. rest.	R
35	May 17	10.30	Right.	11.00	12.48	11.30	12.74	12.61	105	19.40	6.79	62	24	½ hr. rest.	Dr. D.
36	June 6	10.40	Left.	11.00	10.75	11.30	11.15	10.95	94.5	17.50	6.55	70	—	½ hr. rest.	Miss S.
37	June 8	7.15	Left.	7.30	10.40	7.45	10.32	10.36	103	19.04	8.68	78	—	Before getting up.	Dr. P.
38	June 13	7.00	Left.	7.15	11.52	7.25	11.56	11.54	110	20.37	8.83	70	14	Before getting up.	Dr. M.

* Determination of the total oxygen-combining power of the blood done by the Van Slyke apparatus.

TABLE III.

Determinations of Oxygen in Venous Blood Kept for Varying Periods under a Layer of Mineral Oil.

Name.	No. in Tables I and II.	Bleeding.		Determination.		Oxygen content of venous blood.	Remarks.
		Date.	Hour.	Date.	Hour.		
		1917		*1917*		*vol. per cent*	
L.	2	Jan. 26	3.20 p.m.	Jan. 26	3.25 p.m.	13.95	5 min. in laboratory.
				" 26	5.25 "	14.24	3 hrs. in refrigerator at 6°.
				" 27	12 n.	14.20	18½ " " " " 6°.
				" 27	3.30 p.m.	12.28	3½ " " laboratory " 24°.
L.	3	Feb. 27	4.30 p.m.	Feb. 27	4.35 p.m.	14.50	5 min. in laboratory at 24°.
				" 27	5.10 "	14.50	35 " " " " 24°.
				" 28	10.30 a.m.	14.60	41 hrs. in refrigerator " 6°.
L.	6	Jan. 31	12.20 p.m.	Jan. 31	12.40 p.m.	15.80	20 min. in laboratory at 23°.
				" 31	3.15 "	15.75	2½ hrs. " " " 23°.
W.	(2) From another publication.	Mar. 24	12 n.	Mar. 24	2.30 p.m.	7.68	2½ hrs. in laboratory at 23°.
				" 24	2.50 "	7.66	20 min. " " " 23°.
				" 27	3.40 "	11.11	73 hrs. in refrigerator " 6°.
L.	(1) From another publication.	July 2	2.00 p.m.	July 2	2.10 p.m.	17.14	10 min. in laboratory at 25°.
				" 2	2.30 "	17.32	10 " " " " 25°.
				" 9	3.00 "	19.05	7 days in ice box.
L.	(2) From another publication.	July 3	9.50 a.m.	July 3	10.10 a.m.	17.63	20 min. in laboratory at 26°.
				" 3	10.30 "	17.86	20 " " " " 26°.
				" 7	2.00 p.m.	18.64	4 days in ice box.
T.	(4) From another publication.	July 13	9.30 a.m.	July 13	11.10 a.m.	13.83	1½ hrs. in laboratory at 27°.
				" 13	3.40 p.m.	13.99	4½ " " ice box.
				" 13	9.30 "	13.82	5 " " laboratory.

consumption the oxygen unsaturation rather than the oxygen content of the venous blood. The average value of the oxygen unsaturation for Case 1 is 5.5 volume per cent, the minimum being 2.70, the maximum 8.95. The average for the eighteen determinations on Cases 2 to 12 is 6.0 volume per cent, with a minimum of 3.00, a maximum of 8.83 (Table IV).

The determinations are too few to allow an interpretation of the different causes of the variations.[8] Series of determinations on different individuals under different conditions might throw light upon that problem. There is generally a decrease in the oxygen unsaturation with increasing pulse rate, but it is by no means invariable. It is worth mentioning that a value for the oxygen unsaturation of more than 8 volume per cent is found in only four instances, in all of which the blood was drawn in the morning, a few seconds after the subject awoke. In other words, the highest degree of unsaturation is found under circumstances where the metabolism is lowest, when the individual usually has his lowest pulse rate, and when all exciting impressions have been excluded for a considerable time.

In some instances it has been impossible to do the blood analysis immediately after a bleeding. In order to find out how long, and under what conditions the blood samples can be stored, experiments have been done as shown in Table III.

The figures show that the blood can be kept in an ice box at a low temperature (6°C.) for a considerable length of time (at least 24 hours) before any appreciable changes take place. After a certain time the blood will absorb oxygen through the oil and the values will increase. The opposite happens when the blood is kept in the laboratory. We cannot expect it to keep constant more than 2 hours. After that interval the oxygen content diminished rapidly, probably on account of bacterial action.

[8] A series of papers in which the technique described in this paper is applied to the clinical study of patients with heart disease will be published in *The Journal of Experimental Medicine*.

SUMMARY.

1. A report is made of a series of determinations by the Van Slyke method of the oxygen in the blood drawn from the vena mediana in normal resting individuals.

2. A procedure for drawing the blood without stasis or absorption of air has been devised.

3. The difference between the total oxygen-combining power of the hemoglobin and the oxygen in the venous blood is calculated. This figure is termed the oxygen unsaturation of the venous blood.

4. The results are the following:

TABLE IV.

No. of individuals.	No. of determinations.	Oxygen content of venous blood. Vol. per cent.			Oxygen unsaturation of venous blood. Vol. per cent.		
		Maximum.	Minimum.	Average.	Maximum.	Minimum.	Average.
12	38	17.98	9.55	13.6	8.95	2.70	5.8

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ADENINE AND GUANINE IN COW'S MILK.

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(Received for publication, November 19, 1917.)

In the course of some work on the isolation of the antineuritic substance present in cow's milk, we incidentally discovered that this food contains fair amounts of adenine and guanine. Inasmuch as we were unable to locate any reference in the literature to such findings, we present this brief note, which may be of interest in connection with the biochemistry of milk for the following reasons: (1) milk is usually considered to be practically purine-free and has been widely used in metabolism experiments as a purine-free diet; (2) milk was used as a diet in experiments to determine whether or not the animal body can synthesize purines and nucleic acid from purine-free food; (3) the mammary glands may act under certain conditions as an excretory organ for metabolism products, drugs, and poisons.

Concerning the occurrence of purines in cow's milk, Schmidt-Mülheim (1883) claims that he detected free hypoxanthine. Burián and Schur (1897) state that they did not succeed in isolating uric acid from cow's milk; however, the presence of xanthine bases was established by means of the silver precipitate method. The following figures are given: 1 liter of cow's milk yielded approximately 0.005 gm. of purine nitrogen in the silver purine precipitate.

One grave objection can be raised to the method employed by these investigators; namely, the use of $AgNO_3$, a reagent which is known to deaminize amino-purine unless it is used carefully. It is possible that the hypoxanthine which was isolated was in reality due to the deamination of adenine primarily contained in the milk.

EXPERIMENTAL.

The milk was obtained from a herd of Jersey cows and represented a mixed sample. 100 liters of milk were worked up in the following manner: The whole milk, after a thorough shaking, was made slightly acid with acetic acid. The curdled milk was then filtered by suction. To the filtrate an equal volume of 95 per cent alcohol was added and again filtered. This seemed to remove most of the remaining protein and a clear transparent liquid was the result of this last operation. The addition of the alcohol also served a second purpose. As the fluid had to be evaporated to a very small volume *in vacuo*, the addition of alcohol lowered the boiling point of the solution, thus preventing overheating. After evaporating the solution at 45°C. *in vacuo* to about 5 per cent of its original volume a thick white liquid remained. This residue contained fats, milk sugar, lipoids, traces of protein, as well as inorganic salts. The fats and lipoids were removed by repeated extraction with ether, and the protein was removed by a single filtration. The remaining liquid was acid in reaction (acetic), yellowish green in color, and had a syrupy consistency. After standing for 24 to 48 hours, crystals began to appear and after about 2 weeks (depending on concentration) the crystallization became complete. The crystals were lactose. For the sake of convenience 8 liters of milk were worked up in this manner daily. The final volume of each lot of milk amounted to from 350 to 400 cc. The various portions were united and treated with a concentrated aqueous solution of either silver acetate or nitrate. One-fourth of the entire solution (1,500 cc.) was thus treated with Ag acetate in excess. The remainder was precipitated with an excess of silver nitrate. The precipitate caused by the addition of the silver solutions to the milk residue was necessarily divided into two lots; silver purine precipitate from $AgNO_3$ and silver purine precipitate from silver acetate. Both precipitates were filtered off separately and washed well with cold water.

Treatment of Silver Purine Fraction.

The $AgNO_3$ purine precipitate was washed off the filter, suspended in water, to which sufficient HCl had been added to

transform all the silver into AgCl. The latter was filtered off and the filtrate was carefully evaporated to a small volume *in vacuo* at low temperature in order to protect the aminopurines from the destructive action of the nitric acid. The remaining solution was made alkaline with ammonia in order to precipitate the guanine. The guanine settled out as a gelatinous mass, which was redissolved in dilute HCl and reprecipitated with NH_4OH. After drying this precipitate, an amorphous white mass remained.

The filtrate from the original guanine precipitate was treated with CO_2 in order to remove the adenine. This method did not prove satisfactory, so the solution was slightly acidified with HCl and treated with a saturated solution of picric acid in water. After some standing the resulting yellowish brown precipitate was filtered off and recrystallized from water. It gave all the tests for adenine picrate.

The silver acetate purine fraction was treated in the same way and corresponding amounts of guanine and adenine picrate were obtained.

The total amount of adenine picrate from both fractions after recrystallization amounted to 1.141 gm. (0.72 gm. from $AgNO_3$; 0.421 gm. from Ag acetate) or 0.498 gm. of adenine. The total guanine collected (recrystallized) was 1.09 gm.

Identification of Guanine.

The guanine recovered from the purine fraction after reprecipitation from acid solution by NH_3 and drying was a white amorphous powder. It was converted into its different salts as follows.

Guanine Sulfate.—A fraction of a gram of the powder was dissolved in dilute H_2SO_4 (warm) and allowed to stand over night on ice. The following morning long white rhombic needles had formed. The crystals were filtered off, dried *in vacuo* to constant weight, and then a melting point was taken. The crystals lose their water of crystallization at 118–120°. No definite melting point was obtained.

Guanine Picrate.—0.3 gm. of guanine was dissolved in dilute HCl and treated with a saturated aqueous solution of picric acid.

A substance crystallized out in the form of clusters of needles, only light yellow in color. The crystals were filtered off and dried at 100°. The substance had no true melting point but decomposed at 190–200°.

0.1509 gm. of the picrate yielded on analysis 0.0422 gm. N or 29.29 per cent. Guanine picrate contains 29.41 per cent N.

Guanine Hydrochloride.—A small amount of the guanine obtained from milk was dissolved in dilute HCl. On standing at a low temperature crystals formed in the shape of needles. These were filtered off and dried as well as possible in the desiccator over concentrated H_2SO_4. The substance did not have a definite melting point.

0.1212 gm. of the substance yielded 0.0457 gm. N or 37.52 per cent. N calculated for guanine HCl = 37.26 per cent.

Identification of Adenine.

Adenine Picrate.—Adenine picrate as obtained in the way previously described was dried at 105° and gave a melting point of 278–281°.

0.0891 gm. of the picrate yielded 0.0276 gm. N or 30.98 per cent. N calculated for adenine picrate = 30.82 per cent.

The picrate was sparingly soluble in hot water, practically insoluble in cold water. It crystallized in yellow needle-form.

Recovery of Adenine from Adenine Picrate.

From the adenine picrate the free adenine was obtained by treating the picrate with HCl and toluene-ether. The major portion was lost in this operation, but a small percentage of the adenine reclaimed had the following properties.

Melting point, 350–360°; Kjeldahl nitrogen, 0.0722 gm. Adenine gave 0.0372 gm. N or 51.52 per cent; N calculated for adenine = 51.85 per cent. The substance was recovered in the form of white needles.

SUMMARY AND DISCUSSION.

As will be seen from the data reported, cow's milk contains small amounts of both guanine and adenine. 1 liter of milk contains at least 5 mg. of adenine and about 10 mg. of guanine. These values may be considered as minimum values, as the method of isolation of these amino-purines is by no means quantitative. It is interesting to compare these figures with those of Krüger and Salomon (1898–99) dealing with the adenine and guanine content of normal human urine. These authors found in 10,000 liters of urine 3.54 gm. of adenine, no guanine, 10.11 gm. of xanthine and 8.50 of hypoxanthine (accordingly, 1 liter of urine contains 0.35 mg. of adenine and no guanine). The question as to whether the purines found in milk are derived from the blood purines or whether they are formed from the breaking down of the nucleic acid in the mammary gland is still left open.

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THE NUTRITIVE PROPERTIES OF KAFIRIN.

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(Received for publication, November 22, 1917.)

Agriculturists have known for years that the sorghum grains are peculiarly adapted to regions of light or insufficient rainfall, and for that reason they have urged that larger areas in the semi-arid regions be devoted to the culture of kafir, feterita, and milo. In view of the drouth-resisting properties of these grains, and their increasing economic importance, it seemed desirable to investigate more closely their nutritive value.

Such knowledge as we have of the individual proteins of kafir is largely due to Johns and Brewster (1916). These authors found that most of the nitrogenous matter of kafir, about 67 per cent of the total protein, was soluble in alcohol, and they coined the term *kafirin* for this particular protein. They made a careful chemical examination of the material, and for convenience, some of their results are included below.

Analysis of an average of thirteen samples of kafirin gave: C, 55.19 per cent; H, 7.36; N, 16.44; S, 0.60; O, 20.41. The authors also determined the percentage of diamino-acids in kafirin by the Van Slyke method, with the following results: arginine, 1.58 per cent; lysine, 0.90; histidine, 1.00; and tryptophane, present.

Because kafirin forms such a large proportion of the protein of kafir, its nutritive properties were studied in some detail.

A basal ration[1] was prepared in which, for all practical purposes,

[1]

	12 per cent kafirin.	15 per cent kafirin.
	gm.	*gm.*
Protein	126	157
Protein-free milk	257	257
Butter	300	300
Starch	295	265
Agar	20	20

kafirin formed the sole source of protein supply. In all cases this diet resulted in nutritive failure (Chart 1), and accordingly kafirin was supplemented with other deficient proteins, gliadin and gelatin, in the hope that some clue might be obtained to the amino-acid deficiencies of kafirin. These proteins were added to the basal ration, in the proportion of 100 gm. supplement to each 1,000 gm. of the ration. The addition of gliadin, for a short time at least, barely sufficed to maintain the live weight of the animals; but the addition of gelatin enabled them to grow slowly. Both proteins were then added to the basal ration (Chart 2) in the proportion of 50 gm. of each to 1,000 gm. of the ration, with the result that apparently the animals grew still more rapidly than they did when gelatin alone was added. The data are illustrated graphically in Charts 1 and 2.

It is evident, taking the period as a whole, that the rats receiving both protein supplements grew more rapidly than the rats receiving only gelatin. Isolated periods could be selected in which the animals of this latter lot grew as rapidly as the others, but probably no especial significance should be attached to that point. Two or three obvious explanations for the behavior of these rats could be advanced, but the theory chosen tentatively was that there are at least two limiting factors in kafirin as a source of protein. Gelatin supplies one, and gliadin the other.

The most conspicuous advantage of gelatin over gliadin as a source of amino-acid supply is its much larger percentage of lysine. In other respects, however, gelatin is decidedly deficient, as it lacks tyrosine, cystine, and tryptophane. Gliadin, on the other hand, is deficient in lysine, but contains the other three amino-acids in fairly satisfactory proportions. It seemed logical therefore to assume that lysine is the first limiting factor in kafirin, and that the second is one of the three other amino-acids mentioned, tyrosine, cystine, or tryptophane.

Some of the earlier work concerning the rôle of tryptophane in nutrition has been summarized in an earlier paper (1917) from this laboratory. The important rôle that cystine plays in nutrition has been demonstrated by Osborne and Mendel (1915). Their data show that when casein is the only protein in the ration cystine under certain conditions becomes a limiting factor. According to Mathews (1915) casein contains 0.8 per cent of sulfur,

while according to Johns and Brewster (1916) kafirin contains only 0.6 per cent of that element. It is an assumption to calculate that all the sulfur of these proteins is cystine sulfur, but the data make it seem possible that the amount of cystine in kafirin is well below the optimum.

The statement is frequently encountered in the literature that tyrosine is an essential amino-acid. Totani (1916) believes that tyrosine is not necessarily essential, and apparently the facts in the case have not been finally established.

In an effort to investigate the deficiencies of kafirin more definitely, the amino-acids[2] mentioned were added directly to the basal rations. These additions were made singly or collectively as follows: lysine, cystine, lysine plus cystine, lysine plus tyrosine, lysine plus tryptophane.

The lot receiving the basal ration plus lysine grew slowly, in accordance with our expectations. Evidently lysine (Chart 2) is the first limiting factor in kafirin. Accordingly, in our other feeding trials (Chart 3), lysine was always made a part of the basal ration. The further addition of tryptophane to the diet did not increase the rate of growth, and a similar addition of tyrosine (Chart 4) was equally ineffective. When cystine was superimposed on this ration, however, the animals grew somewhat more rapidly. Small variations might seem insufficient to establish this point, but the unanimity of the results seems conclusive. Furthermore, the marked acceleration in growth that follows the addition of cystine after it has been withheld for some time seems even more conclusive. Accordingly, our interpretation of the data is that cystine is the second limiting factor in kafirin. Charts 3 and 4 give the data in greater detail.

[2] In some cases the tyrosine used was a Kahlbaum preparation, in others it was prepared by the author. The cystine, tryptophane, and lysine were prepared in this laboratory.

The amount of lysine added varied from 3 to 6 per cent of the kafirin in the ration, but the larger amount seemed no more effective than the smaller. The cystine formed 5 per cent of the kafirin, and enough tyrosine was added to equal 4 per cent of the protein.

The Lysine Requirement of Immature Animals.

The data bring out one fact of general application; namely, that lysine is indispensable, even for the maintenance of young animals. This point has been in doubt for some time because of the difficulty in proving that a diet contains no lysine. Gliadin was formerly thought to lack that particular amino-acid, and because of that belief, earlier experimental work on the nutritive properties of gliadin was assumed to have special significance. Osborne and Mendel (1914) stated that rats maintained their live weight unchanged, but failed to grow when gliadin formed the sole source of protein in the ration. Their interpretation was that lysine is necessary for growth, but is not necessary for maintenance. When somewhat later the presence of lysine in gliadin was established, they (1916) recognized the element of doubt attached to their earlier pronouncement. The behavior of animals receiving their protein in the form of zein has also been interpreted as an indication that lysine is not required for the maintenance of nutritive equilibrium. Willcock and Hopkins (1906) found that mice receiving zein as the sole source of protein died in less than 2 weeks. Those receiving zein and tryptophane were active after 16 days of zein feeding, but had lost weight. Wheeler (1913) obtained similar results, but even after fortifying the zein with tryptophane the animals constantly lost weight. Similar results are recorded by Osborne and Mendel (1914) in their series of experiments. Their animals receiving a zein plus tryptophane diet were at a nutritive standstill or lost weight, though they grew rather rapidly on the further addition of lysine.

The precise status of lysine in nutrition remained uncertain, however, because of the difficulty in estimating or even detecting small quantities of that amino-acid. Recently Osborne, Van Slyke, and collaborators (1915) published data indicating no inconsiderable quantity of lysine in gliadin. The method of Van Slyke yielded an average result of 1.33 per cent, and the direct method of Kossel and Kutscher resulted in the recovery of 0.64 per cent of the gliadin as lysine. There is no doubt, therefore, that gliadin contains approximately 1 per cent of lysine, while kafirin, according to Johns and Brewster (1916),

contains 0.90 per cent. On the other hand, such evidence as is available points strongly to the complete absence of lysine from zein. Osborne and Leavenworth (1913) found no trace. Furthermore, according to a theory advanced by Van Slyke and Birchard (1914), proteins containing lysine possess free amino groups that react with nitrous acid; but zein gives no evidence of such a reaction. Gliadin, on the contrary, according to their data yields 1.10 per cent of the total nitrogen as free amino nitrogen.

Although this evidence may be questioned, yet such weight as it has is all against the presence of lysine in the zein complex. If the facts deduced from zein feeding contradict our findings while feeding kafirin, we have no explanation for the discrepancy.

SUMMARY.

Lysine is the first limiting factor in kafirin and cystine is the second.

Lysine is indispensable for the maintenance of young animals.

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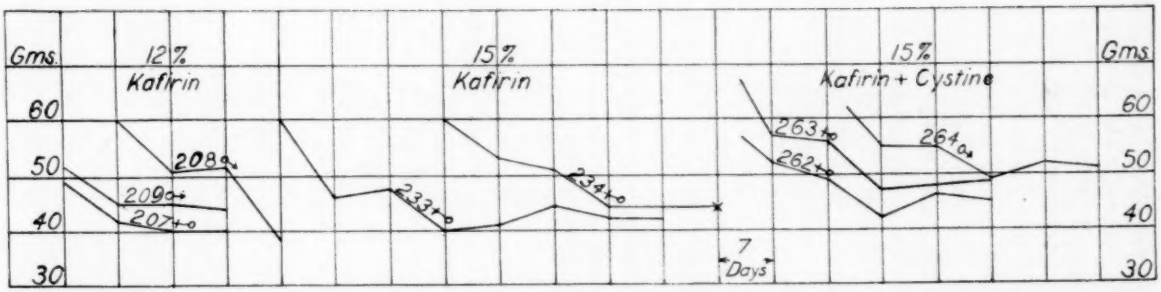

CHART 1. One lot of animals received 12 per cent, another 15 per cent kafirin.[1] The inadequacy of this protein is indicated by the behavior of these rats. They lost weight and manifested the usual symptoms of malnutrition, such as diseased eyes, roughened coat, and muscular weakness. Rat 234 died at the point indicated by X. The lot receiving cystine as a supplement is included here inasmuch as these animals had obviously not been affected in any way by that addition to the ration.

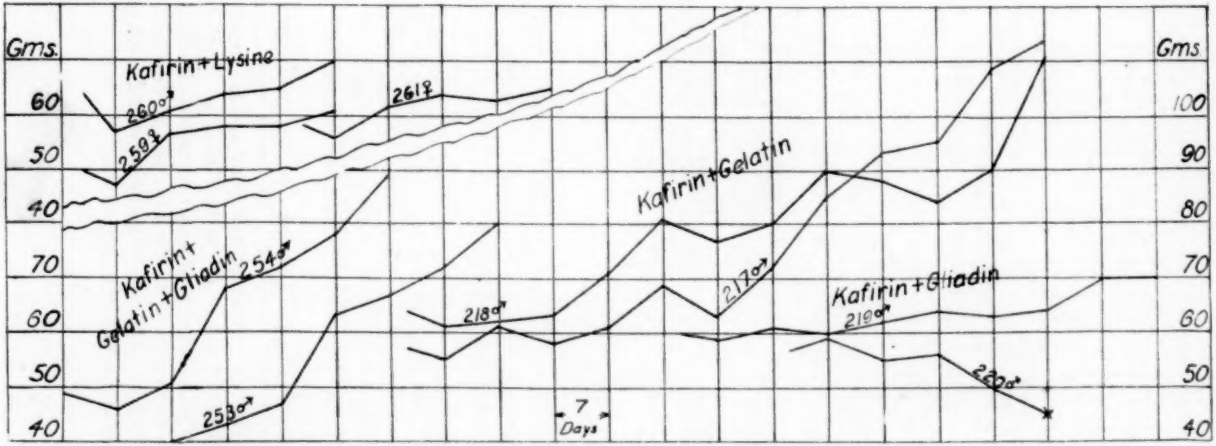

CHART 2. All animals received the same basal ration,[1] 15 per cent kafirin. The addition of gelatin as a supplement (10 per cent of the entire ration) permitted fairly rapid growth. Gliadin in equal quantity was much less effective. One rat receiving this adjuvant grew very slowly, the other died at the point indicated by X. When the gelatin and gliadin diets were combined in equal proportions and fed to another group of rats, growth occurred more rapidly than on the larger quantity of gelatin given alone.

These facts seemed to indicate that lysine is the first limiting factor in kafirin, and when this amino-acid (5 per cent of the protein) was added to the basal ration, that inadequate diet was converted into one permitting a slow rate of growth.

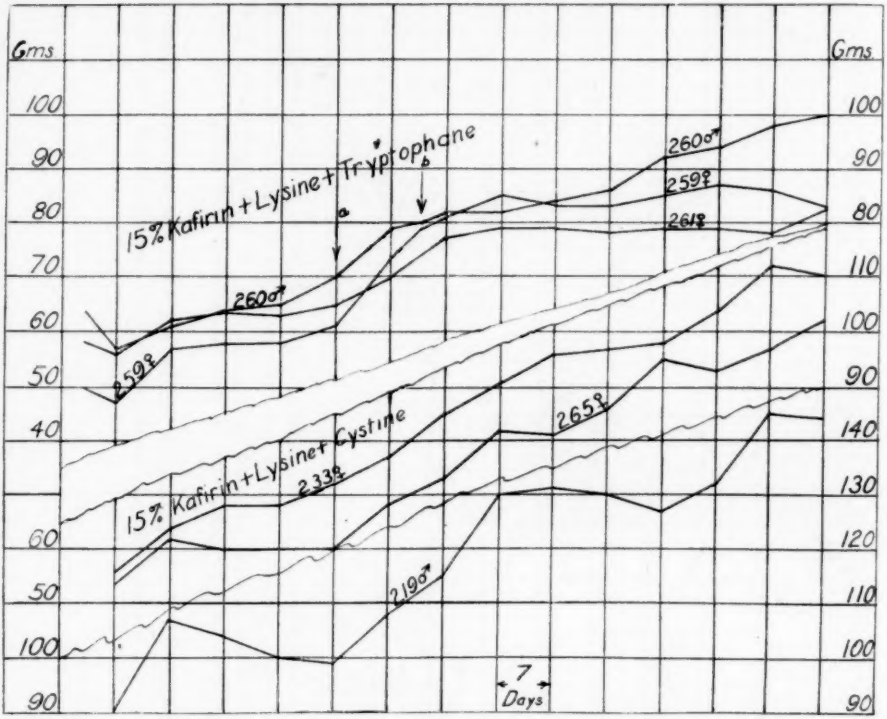

CHART 3. All these animals received a ration containing 15 per cent kafirin,[1] and lysine. Three of them, Rats 259, 260, and 261, received in addition tryptophane and cystine at the point shown by *a*. At point *b*, the cystine feeding was discontinued and thereafter these rats received as amino-acid supplements only lysine and tryptophane. The other three animals, Rats 219, 233, and 265, received cystine in addition to the lysine at all times.

The effects of cystine feeding are not marked, but the lot receiving this amino-acid made a distinctly greater gain than the other. The males normally grow faster than the females, yet Rat 260 on the tryptophane ration, a male, grew somewhat more slowly than either of the females receiving the cystine diet. For a short time, the tryptophane lot received cystine, and it seems significant that in this brief period they should exhibit such a marked increase in the rate of growth. If the cystine feeding had been omitted, it seems probable that these rats would have made a still more unfavorable showing as compared with the cystine lot.

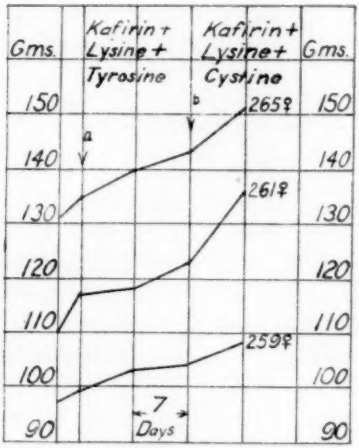

CHART 4. The data shown in this chart are not complete, as the investigation was unavoidably interrupted. In spite of the fact that the period of these feeding trials was short, however, we believe that it was sufficiently long, at least when considered in relation to Chart 3, to admit of a final conclusion.

Rats 259 and 261 had been receiving 15 per cent kafirin and lysine[2] plus tryptophane,[2] and Rat 265 had been on a diet of 15 per cent kafirin plus lysine plus cystine.[2] At the point indicated by *a* all were changed to a ration of 15 per cent kafirin plus lysine plus tyrosine.[2] The rate of growth of the animals was apparently not affected, certainly it was not increased. At point *b* tyrosine was replaced by cystine, and the rate of gain was increased in all cases. In two instances it was marked.

THE OCCURRENCE OF LICHENASE IN THE DIGESTIVE TRACT OF INVERTEBRATES.*

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No animal enzyme has yet been found by which the hydrolysis of lichenin, the peculiar dextran of *Cetraria islandica*, may be accomplished. Of the vegetable enzymes, Saiki (1) has reported that the taka-diastase of *Eurotium oryzæ* and the inulase of *Aspergillus niger* were active in the hydrolysis of lichenin, while malt diastase was inactive, as previously observed by Brown (2). Examination of human saliva (1, 2, 3) and gastric juice (3), of extracts of the pancreas of the dog (1, 3) and ox (3), and of the intestine of the pig (1) for the presence of a lichenase have uniformly yielded negative results. In view of this resistance to hydrolysis by the body enzymes of the higher animals, as pointed out by Nilson (3), the problem of the utilization of lichens by animals, such as the reindeer, and in human dietaries offers many difficulties. Bacterial decomposition might be expected to play a rôle here, but according to Saiki (1) cultures of *Bacillus coli* do not form reducing sugars from lichenin.

In connection with a study of the fresh water crayfish, it was observed that extracts of the hepatopancreas possessed marked hydrolytic properties towards solutions of lichenin, and further investigations of certain other invertebrates disclosed the existence of lichenase in these animals. Accordingly, a systematic search for the presence of lichenase in the alimentary tract of such vertebrate and invertebrate forms as were available was undertaken.

The lichenin used as a substrate was derived from three sources: (1) crude extracts of Iceland moss, (2) lichenin obtained from

* A preliminary account of this investigation was presented before the Society for Experimental Biology and Medicine, New York, Dec. 20, 1916.

a chemical supply house, and (3) lichenin prepared and purified in this laboratory by the method of Berg (4). The first preparation was used only in comparison with one of the other preparations. Tests for isolichenin made on the other two preparations were uniformly negative (color with iodine). No differences were noted in these two preparations when used as substrates in the enzyme experiments. As controls of the activity of the extracts under investigation, freedom from bacterial decomposition, etc., tests were also carried out on other typical carbohydrates, inulin, raffinose, sucrose, and starch, for the presence of other enzymes. In view of the widespread distribution of amylase, it was thought that the absence of amylase would be indicative of loss of enzyme activity in the preparation of the extract. As an additional control, the tissue extract itself was incubated with an amount of water equal to the volume of the carbohydrate solution used. This was done in order to test for the presence of any polysaccharide (*e.g.*, glycogen) which might be present in the tissue and yield a reducing sugar on autolysis. Only in the case of two of the mollusks studied (*Unio gibbosus* and *Lampsilis luteola*) was there found evidence of the presence of such a carbohydrate. In the experiments with these species comparative tests showed the presence of enzymes which acted upon the other sugars also. Control tests with boiled extracts were also made in each case. All experiments were carried out at room temperature, at about 21°C. Toluene was used as a preservative. 0.5 per cent solutions of lichenin and 1 per cent solutions of the other carbohydrates were tested. Tests for reducing sugars[1] with Fehling's solution were made after 1, 3, 5, and 7 days.

The hepatopancreas was chosen for investigation in most of the invertebrates studied (Nos. 4 to 18, Table I). In the small animals (1, 3, 20, Table I) the entire organism was used, while in the earthworm and grasshopper the entire alimentary canal was removed for examination. The animals were killed with ether, the tissues removed immediately, washed, and ground up

[1] In a number of experiments the reducing sugar was further identified by the formation of the osazone. An osazone crystallizing from the hot solution, and identified microscopically as glucosazone, was obtained from the tubes showing reduction, but never from the control tubes.

with sand and water in a mortar. The extracts were then filtered through cotton. Extracts of the organs of the pig, sheep, and dog could not be made until from 12 to 24 hours after their removal from the body.

The detailed results are shown in the tables. *An enzyme capable of hydrolyzing lichenin with the formation of a reducing sugar was found to be present in the alimentary system of each of the twenty species of invertebrates examined* (*Table I*), *while in no case was such an enzyme observed in the alimentary tract of the vertebrates under investigation* (*Table II*). In every instance the extracts of the vertebrate tissue were active, as evidenced by their strong amylolytic properties. This inactivity of extracts of the organs of the vertebrate alimentary system is in agreement with the limited number of observations of earlier investigators. Von Tschermak (5), however, has reported the presence of lichenase in the pancreas or intestine of half the number of normal rabbits examined in connection with his studies on adaptation of enzymes to diet. Glycerol extracts of the organs were used and the fermentation test was made as a criterion of the absence or presence of a monosaccharide after incubation of the extracts of the tissue with the lichenin. Differences in technique may account for the variations between his work and that of other experimenters.

That lichenase was not uniformly distributed throughout the whole invertebrate organism was demonstrated by tests carried out on the extracts of the muscle tissue of the crayfish (*Cambarus virilis*). Repeated examination of muscle extracts of this species gave no evidence of the presence of lichenase, although extracts of the hepatopancreas of the same individuals showed marked action on lichenin. It has been suggested by von Tschermak (5) that the enzymes for the hydrolysis of lichenin and inulin are closely associated or may even be identical (inulolichenase). The inulase of *Aspergillus niger* is also able to effect the hydrolysis of lichenin (1). In the present series of experiments there is no evidence of any close association between inulase and lichenase. While lichenase was found to be present in the alimentary tract of twenty different species, inulase was observed in only one instance, in the experiments with *Lampsilis luteola*. There is no apparent relationship between the occur-

TABLE I.

Phylum.	Species.	Inulin.	Lichenin.	Raffinose.	Sucrose.	Starch.
Porifera.	1. Marine sponge (?)		+			+
Annelida.	2. Earthworm (*Helodrilus caliginosus*).	−	++	−	+	+++
"	3. Leech (*Dina sp. ?*).		+			+++
Echinodermata.	4. Starfish (*Asterias ochracea*).*	−	++		+++	
"	5. Starfish (*Asterias forbesi*).	−	++	−	+++	+++
"	6. Sea urchin (*Strongylocentrotus dræbachiensis*).	−	++	−	+++	+++
Mollusca.	7. Black leather chiton (*Katharina tunicata*).*	−	+		− ✓	
"	8. Giant chiton (*Cryptochiton stelleri*).*	−	++		− ✓	
"	9. Mussel (*Unio gibbosus*).		++		++	
"	10. Mussel (*Lampsilis luteola*).	+(?)	++	+(?)	++	++
"	11. Mussel (*Alasmidonta marginata*).	−	++	−	+++	
"	12. Snail (*Planorbis trivolvis*).	−	+++	−	+++	+++
"	13. Snail (*Physa gyrina*).		++			+++
Arthropoda.	14. Crab (*Hemigrapsus nudus*).*	−	++		+++	
"	15. Crab (*Cancer productus*).*	−	+		+++	
"	16. Mud crayfish (*Upogebia sp. ?*).*	−	++		+++	
"	17. Shrimp (*Pandalus sp. ?*).*	−	++		++	
"	18. Fresh water crayfish (*Cambarus virilis*).	−	++	++	+++	+++
"	19. Grasshopper (*Melanoplus differentialis*).	−	+++	+++	+++	+++
Protochordata.	20. Sea squirt (*Cynthia sp. ?*).		+			+

* The experiments with these species were carried out during the summer of 1916 at the Puget Sound Station, Friday Harbor, Washington. Thymol was used as a preservative in these experiments instead of toluene.

TABLE II.

Vertebrates in Which the Absence of Lichenase Was Uniformly Demonstrated with Organ or Tissue Investigated.

Class.	Species.	Organ.
Pisces.	Gold fish.	Alimentary canal.
Amphibia.	Frog (adult and tadpole).	" "
Reptilia.	Horned toad.*	" "
"	Garter snake.	" "
"	Turtle (two species).	Pancreas, upper portion of small intestine.
Aves.	Domestic fowl.	" " "
Mammalia.	Rabbit.	" " "
"	Pig.	Pancreas.
"	Sheep.	Small intestine.
"	Dog.	Pancreas.
"	Man.	Saliva.

* Obtained through the courtesy of Professor A. O. Weese, of the University of New Mexico.

rence of these two enzymes in the organism of the invertebrates studied.

One other point in connection with the presence of enzymes other than lichenase is of interest. In the hepatopancreas of the two chitons (Nos. 7, 8, Table I) sucrase was absent. Since this enzyme is so widespread in its distribution examinations were made repeatedly on these species with the same negative results as far as sucrase was concerned. Of the sixteen species of invertebrates examined for this enzyme, these were the only two in which the presence of sucrase could not be demonstrated.

It was realized that with the preservative used, toluene, bacterial action was not necessarily completely inhibited. In view, however, of the uniformity of the results, positive with invertebrates, and negative with vertebrates, hydrolysis of the lichenin can hardly be ascribed to the action of the bacteria, since if bacterial enzymes are concerned there is no reason why vertebrate extracts should not be contaminated with bacteria as frequently as invertebrate extracts. Moreover, it would be difficult to understand why inulin or raffinose should not be attacked as well as lichenin since many strains of *Bacillus coli*, one of the prevailing types of organisms of the alimentary tract, ferment

raffinose and inulin readily (6). Also according to Saiki (1), *Bacillus coli* does not attack lichenin. Experiments were conducted to determine the degree of bacterial contamination in the experimental tubes in which toluene was present as a preservative. The alimentary canals of a number of grasshoppers were removed, macerated, and tests on lichenin made as before with the usual controls. After 24 hours, reducing sugar was present in considerable amounts. A bacteriological examination of the contents of the tubes was then made.[2] Agar plates showed four to eight colonies per cc. after 48 hours' incubation at 37°C. Plates from the boiled controls gave similar results. The prevailing organism was a long spore-forming bacillus of the *subtilis* group. The number of organisms can hardly have been great enough to cause the strong hydrolysis observed, especially since the unattacked control tubes showed equal bacterial contamination. Extracts of the alimentary canal of grasshoppers were also thoroughly mixed with chloroform and toluene and allowed to stand 12 hours with frequent shaking. The clear extract removed with a sterile pipette showed marked hydrolytic action on lichenin.

Chemical studies have led to few clear-cut distinctions between vertebrates and invertebrates. That a line of demarcation between the two exists in a difference in the creatine metabolism seems probable. No invertebrate tissue has been found to contain creatine, while from the muscle of the lamprey-eel, the most primitive vertebrate examined, creatine was easily isolated (7). The results of the present study on a limited number of forms suggest a second distinction between vertebrates and invertebrates in the presence of lichenase in the alimentary tract of the former. It is hoped that examination of more primitive vertebrates and higher invertebrates may show whether this difference is general or not.

[2] The bacteriological examinations were carried out by Dr. J. A. Sperry, of the Department of Bacteriology, to whom we take this opportunity of expressing our indebtedness for his cooperation.

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4. Berg, T., *Russ. Z. Pharm.*, 1873, 129, 161; cited in *Jahresb. Chem.*, 1893, 848.
5. Von Tschermak, A., *Biochem. Z.*, 1912, xlv, 452.
6. Cf. Kligler, I. J., *J. Infect. Dis.*, 1914, xv, 187.
7. Mellanby, E., *J. Physiol.*, 1907–08, xxxvi, 447. Wilson, D. W., *J. Biol. Chem.*, 1914, xviii, 17.

THE AMOUNT AND THE DISTRIBUTION OF CREATININE AND CREATINE IN NORMAL HUMAN BLOOD.

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Of the non-protein constituents of blood, some, such as potassium,[1] phosphoric acid,[1, 2] lecithin,[3] free cholesterol[3, 4, 5] and amino-acids,[5, 7, 8] are localized to a greater or smaller extent within the corpuscles; some, such as sodium,[1] calcium,[1] chlorine,[1] cholesterol esters,[5] glucose,[9] and adrenalin,[10] are more abundant in, or even entirely confined to the plasma; some, finally, with urea[6, 11] as their most conspicuous known representative, maintain a practically equal concentration in both. There are few important constituents of which the relative concentration in

[1] Abderhalden, E., Lehrbuch der Physiologischen Chemie, Berlin and Vienna, 1906, 591–593.

[2] Porte, A., *Compt. rend. Soc. biol.*, 1914, lxxvii, 467.

[3] Bloor, W. R., *J. Biol. Chem.*, 1916, xxv, 577.

[4] Hepner, E., *Arch. ges. Physiol.*, 1898–99, lxxiii, 595.

[5] Bloor, W. R., and Knudson, A., *J. Biol. Chem.*, 1917, xxix, 7.

[6] Bang, I., *Biochem. Z.*, 1915, lxxii, 104; 1916, lxxiv, 294.

[7] Bock, J. C., *J. Biol. Chem.*, 1917, xxix, 191.

[8] Wilson, D. W., and Adolph, E. F., *J. Biol. Chem.*, 1917, xxix, 405.

[9] Macleod, J. J. R., Diabetes; Its Pathological Physiology, London, 1913, 31. The distribution of glucose in the blood is, however, still the subject of debate. Quite recently Gradwohl and Blaivas (*J. Lab. and Clin. Med.*, 1916–17, ii, 416), using the method of Lewis and Benedict, found the sugar content of the corpuscles in man to be nearly always the same as that of the plasma.

[10] Stewart, G. N., and Rogoff, J. M., *Proc. Soc. Exp. Biol. and Med.*, 1916–17, xiv, 79.

[11] Marshall, E. K., and Davis, D. M., *J. Biol. Chem.*, 1914, xviii, 53. Karr, W. G., and Lewis, H. B., *J. Am. Chem. Soc.*, 1916, xxxviii, 1615. In fish blood urea is chiefly localized in the corpuscles; see Wilson and Adolph.[8]

corpuscles and plasma is unknown; but to this category belonged until quite recently creatinine and creatine.

The distribution of materials within the blood is not only a matter of considerable interest in itself; it possesses an obvious bearing upon many questions of much wider import. In the transport of materials to and from the tissues every element of the blood may play a part; but their actual passage into or out of the circulation can be effected only through the plasma. Processes like absorption, or excretion, or placental transmission may therefore raise problems for the solution of which a knowledge of concentrations in the blood as a whole is insufficient, and the determination of plasma concentrations, which may be very different, becomes a prime necessity. Creatinine and creatine, in what is already known of their behavior under physiological and pathological conditions, present a number of problems of this description.

For instance, although creatinine is being constantly excreted by the kidney, the laws which regulate its passage through that organ are unknown; any attempt to formulate them presupposes an acquaintance with the variations of the plasma creatinine, which may or may not parallel those of the creatinine of the entire blood. Creatine, on the other hand, in spite of the fact that its concentration in the blood is greater than that of creatinine, is usually absent from the urine of the male adult; but we do not know whether this is because it is localized exclusively in the corpuscles, or because its elimination, like that of glucose and chlorides, is dependent upon the crossing of a certain threshold of concentration in the plasma. The doubt could be resolved, and fresh light might be thrown upon the occasional occurrence of creatinuria, if we had precise information regarding the actual distribution of creatine in the blood. During pregnancy, to take a problem from another field, creatinine and creatine are found in the fetal as well as the maternal blood; and it is natural to inquire to what extent and in what manner these substances share in the transfer of materials from one circulation to another across the placenta. Obviously the question demands for its answer a knowledge of their concentrations in the respective plasmas. Even in pathological conditions a study of the relations under discussion might well reveal something of interest. The accu-

mulation of creatinine in the blood has shown itself to be a useful index of renal insufficiency; but we are as yet unaware whether the excess present in the circulation of a nephritic permeates all the elements of the blood, or accumulates in the plasma alone. If the latter alternative should prove to be correct, the variations of the plasma creatinine in kidney disease would be even more striking than those of the whole blood creatinine, and would form a still more delicate index of the organ's capacity to excrete. A separate study of plasma and whole blood creatinine and creatine in different pathological conditions might even reveal significant variations in the permeability of the corpuscles for these substances.

It was with these considerations in mind that we undertook recently to add creatinine and creatine to the list of blood constituents with more or less definitely established distribution.

In a preliminary communication,[12] in which each aspect of our interest in the problem was distinctly indicated, we have already presented an abstract of our earliest results. This constituted, as far as we have been able to discover, the first published contribution to the subject in relation to human, or indeed mammalian blood; although some data upon the distribution of creatinine and creatine in fish blood were simultaneously communicated by Wilson and Adolph.[8] Among the conclusions which we thought to be justified by our observations the chief was that both in creatinine and in creatine the corpuscles are under nearly all conditions richer than the plasma. So long as the reliability of our methods was taken for granted no other conclusion was possible. A continuation of the work along the original lines did nothing to modify the general tendency of our analytical results, but it gradually engendered doubts concerning their real significance. These doubts were not a little accentuated by the work of Wilson and Plass,[13] who, dealing a little later with the problems upon which we were engaged, confirmed in a general way our view regarding the distribution in human blood of creatine, but upon that of creatinine reached a different

[12] Hunter, A., and Campbell, W. R., *J. Biol. Chem.*, 1917, xxix, p. xviii.

[13] Wilson, D. W., and Plass, E. D., *J. Biol. Chem.*, 1917, xxix, 413.

conclusion. We therefore deemed it necessary, before publishing a fuller account of our work, to satisfy ourselves upon the precise value of the analytical methods which we had employed. The issue of this incidental, but necessary inquiry, the details of which have been recorded in another paper,[14] was such as to necessitate a revision of some of our original conclusions. During its progress we confined our attention, in the accumulation of additional data, almost entirely to the fundamental points of the amount and the distribution of creatinine and creatine in normal human blood. It is to the results bearing upon these points, and to the interpretation of them which now appears to us the most plausible, that the present paper is principally devoted. Other problems, such as the relation of blood creatine to creatinuria, are touched upon only in so far as incidental observations offer occasion. It is hoped that the opportunity may yet arise to carry the inquiry further along each of the lines we have proposed.

Subjects.

The blood specimens, which for our present purpose were assumed to be normal, numbered 60, and were derived from 56 individuals, who fall into three main groups. The first, Group I, consists of 26 male students and instructors, all, as far as could be judged, in perfect health; 2 of these provided three specimens each of blood so that the total number of analyses performed within this group was 30. The second, Group II, includes 20 hospital patients, male and female, mostly convalescent after childbirth or purely surgical complaints, and all free, as far as known, from organic disease. This group is divided into two subgroups; but the distinction between them is one of date only, Group II A having been studied several months earlier than II B. The final group, Group III, is formed by 10 healthy women in a late stage of pregnancy, of whom 1 only was as yet confined to bed.

No subject has been admitted to any of these groups in whom a routine examination revealed evidence of organic disease. In this respect all equally were normal individuals; but of course

[14] Hunter and Campbell, *J. Biol. Chem.*, 1917, xxxii, 195.

the figures yielded by Group I possess a rather better claim than the others to be regarded as standards. The patients of Group II were not exactly in the best physical condition, nor were they living under altogether normal circumstances; while, with reference to Group III, we cannot at present be certain that even a normal pregnancy is entirely without effect upon the relations we are studying. The evidence furnished by Groups II and III is therefore to be taken as suggestive or confirmatory rather than conclusive in itself.

Methods.

The blood was taken from a superficial arm vein by means of a syringe, and its coagulation was prevented by the prompt addition of 20 per cent potassium oxalate in as nearly as possible the proportions prescribed by Folin.[15] The plasma was immediately separated from a portion by centrifugation. While this was being done, the subject was usually required to provide a specimen of urine. The analyses planned were then carried out with the briefest possible delay. They included the determination of total and preformed creatinine in whole blood, plasma, and urine, together with the estimation, by means of the simplified hematocrit of Epstein,[16] of the relative plasma volume in the oxalated blood.[17] For the creatinine and creatine determinations in blood and plasma we employed the methods of Folin,[15] although the technique followed was not always precisely the same. Thus in many of our earlier observations we followed the directions of Myers and Fine[18] in laking the blood before saturating it with picric acid; and in our creatine determinations we did not take the precaution, which we have lately[14] found to be desirable, of so diluting blood and plasma in every case that the same standard could be employed throughout the series. In one respect at

[15] Folin, O., *J. Biol. Chem.*, 1914, xvii, 475.

[16] Epstein, A. A., *J. Lab. and Clin. Med.*, 1915–16, i, 610.

[17] In many cases, but not all, we had sufficient blood to determine also the total non-protein nitrogen. It seemed hardly worth while to report individually the scattered results thus obtained. It may be stated that they ranged from 29 to 40 mg. per 100 cc., and that, as far as they went, they revealed no constant relation to the data for creatinine.

[18] Myers, V. C., and Fine, M. S., Chemical Composition of the Blood in Health and Disease, Cooperstown, N. Y., 1915, 19.

least our practice was absolutely uniform; the creatinine value of each color reaction was obtained by reference to the standard curves which we have recently described.[19] For the determination of creatinine and creatine in urine we usually employed the macro method, in which case the conversion of creatine was accomplished by the procedure of Benedict.[20] In a few instances we utilized the micro method of Folin.[21] The chief purpose of the urine analysis was the detection and measurement of the creatine. The creatinine results, which have yielded little of value in themselves, are recorded chiefly for the sake of completeness.

The urine specimens were subjected also to a routine qualitative examination.

Results.

The analytical results are shown in Table I.[22] They are divided into the groups and subgroups already mentioned, and are arranged within each group so as to present an ascending scale of plasma creatinine. Certain other ways of arranging the results illustrative of special points discussed in the text are employed in the general averages at the end of the table. The corrections introduced into the table are made, as explained later, on the basis of conclusions reached in our critical examination of the methods.[14] The few figures given for creatine of the corpuscles are calculated from the whole blood and plasma creatine in conjunction with the relative plasma volume.

DISCUSSION OF RESULTS.

It has been shown[14] that of the four creatinine determinations required in the complete analysis of blood that of the preformed creatinine of plasma has the best claim to accuracy. It is for this reason that we selected the plasma creatinine to determine the arrangement of our results; and for the same reason we shall discuss its variations before attempting to estimate the significance of our other data.

[19] Hunter and Campbell, *J. Biol. Chem.*, 1916–17, xxviii, 335.

[20] Benedict, S. R., *J. Biol. Chem.*, 1914, xviii, 191.

[21] Folin, *J. Biol. Chem.*, 1914, xvii, 469.

[22] Many of them are taken from the thesis submitted by W. R. Campbell in partial fulfillment of the requirements for the degree of M.D., University of Toronto, 1917.

Plasma Creatinine.

In our preliminary communication[12] we stated that the plasma creatinine of normal individuals is always less than 1.0 mg. per 100 cc. This opinion was based on the nine results which constitute Group II A of the larger series here presented. It is now apparent that these results were by no means completely representative. The figures of Group I show that at least for healthy and active males the ordinary level of the plasma creatinine is somewhat higher than our early estimates. Of the 30 results within that group no more than 8 are below 1.0 mg. A majority (17) are included by the values 1.1 and 1.2. The lowest is 0.80, the highest 1.3. The average of the whole group is 1.09, or in round figures 1.1. These data are furnished by a number of separate individuals (26) sufficiently large to make them fairly representative of their class.

When it was found that Group I, our second series in point of time, gave generally higher values than Group II A, our first, we were inclined for a little to question the correctness of the earlier analyses. We therefore, by way of control, supplemented these at a considerably later date by a further series of eleven determinations upon strictly comparable material. The results, constituting Group II B, do not differ in any essential respect from those of Group II A. One, it is true, shows a value as high as any found even in Group I; but of the remainder none exceeds 1.0 mg., and eight are below it. The results in the two subgroups therefore mutually confirm one another, and form together a sufficiently concordant single series. Taking them as such we find that the plasma creatinine of Group II ranges from 0.70[23] to 1.3, or, neglecting the last as a distinctly aberrant value, to 1.0. The average of all is 0.89, or, if we again exclude

[23] We have in our records one case only where the plasma creatinine appeared to be lower even than the minimum here reported. It was that of a female patient without known organic disease, supposed to be suffering from hysteria, who might fairly enough have been included in Group II. Her plasma creatinine was 0.58. This value remained so unique that, lacking opportunity to confirm it, we have hesitated to place confidence in the correctness of the analysis, and have therefore omitted it from the table.

the maximum from the calculation, 0.86. It is clear that the hospital inmates selected as normal individuals presented in general an unmistakably lower level of plasma creatinine than the active male students.

With respect to the cause or causes of this difference we can only speculate. Group II, unlike Group I, included a considerable proportion of females. Several of its members showed an abnormally high relative plasma volume, possibly indicative of a certain degree of dilution of the blood. Three-fourths of them were confined to bed, and the others were at least leading a sedentary existence. All, it is safe to assume, were eating less than the more actively employed members of Group I, and some, it is known, were on a very restricted diet. It is not yet possible to decide what actual influence any or all of these circumstances may have exerted upon the plasma creatinine of the group as a whole. Certainly neither the first mentioned nor the second will wholly explain its relative lowness. For, while it is true that the average for the females of the group is lower than that for the males, and even that within each subgroup the maximum for women is lower than the minimum for men, yet the males, considered apart, still yield distinctly less creatinine than those of Group I; and, although dilution of the plasma doubtless may diminish its creatinine concentration, there is evidence on the one hand (Cases 58, 59, 60) that it does not always do so, and on the other (Cases 3, 5, 36) that a plasma creatinine below the average may be encountered in blood which is the reverse of hydremic. Of the other two possible factors suggested, low diet and enforced inactivity, the latter is the one which seems the more likely to be of general importance; for the production of creatinine within the body is independent of the diet but intimately related to the condition of the muscular tissues.[24] It may well be assumed that few, if any, of our hospital subjects maintained themselves, while confined to bed, upon the same plane of muscular efficiency as the members of Group I. Provisionally therefore we are inclined to ascribe the low plasma creatinines of Group II partly to the female sex of some of its members, but largely to lack of exercise in its effect upon mus-

[24] Shaffer, P. A., *Am. J. Physiol.*, 1908–09, xxiii, 1.

cular tone. If the associations thus suggested are correct, a low plasma creatinine would be characteristic of such cases as might be expected to have a low creatinine coefficient; and it is of some interest that according to Shaffer[25] there is found under similar conditions a smaller concentration than usual of creatinine in the muscles also. The possible influence of prolonged inaction upon the plasma creatinine could obviously be put to experimental proof; and a further examination of the question is accordingly in contemplation. Meanwhile it may be of practical clinical importance to note that a plasma creatinine of 1.2 mg., which would be perfectly normal in an active subject, already verges upon the pathological for one who is resting in bed.

The idea, suggested by the results within Group II, that females have naturally a lower plasma creatinine than males, receives additional support from the data furnished by the subjects of Group III. These, as already stated, were all females, in whom pregnancy constituted the only deviation from normality. The ten plasmas included in this group contained amounts of creatinine ranging from 0.71 to 1.2, seven of them falling below 1.0 mg., and the average of all reaching only 0.92. These figures certainly occupy a lower general level than those of Group I, with their minimum of 0.80 and their average of 1.1. It may, of course, be objected, that women advanced in pregnancy, even if they are still taking a certain amount of exercise, can hardly be regarded as physiologically comparable with active young men. The comparison, at the end of Table I, of the general average for all males (1.04) with that for all females (0.87) has to be regarded with similar reserve. It is really only within Group II that difference of sex is not obviously complicated by some other circumstance of possible consequence. We are therefore not yet prepared to state with conviction, although we regard it as exceedingly probable, that the sex effect indicated by our figures has an existence independent of all other factors. To determine finally its actual importance, it would be necessary to possess for comparison with the data of Group I a series of observations upon active and healthy young women; unfortu-

[25] Shaffer, *J. Biol. Chem.*, 1914, xviii, 525.

nately the opportunity of collecting such a series has not yet arisen.[26]

There is no evidence in any of the groups that the variations of the plasma creatinine are definitely related to the age of the subject, at any rate within the limits of 12 to 55 years.

Creatinine of Whole Blood and Its Distribution.

In each of the 60 pairs of preformed creatinine determinations included in the table the whole blood yielded a higher value than the plasma. As far, therefore, as the immediate outcome of the analysis is concerned, our original report[12] receives here the fullest corroboration. If the colorimetric method were of the same accuracy in blood as in plasma, it would follow at once that, as we formerly announced, the creatinine of the blood is chiefly concentrated in the corpuscles. Unfortunately the condition of equal accuracy is not fulfilled. We have recently,[14] on a careful examination of the method, convinced ourselves that, as Wilson and Plass[13] had already assumed, the values reported for whole blood are an exaggeration of the truth. The extent of the exaggeration we found to vary with the individual instance and the particular technique employed. With the original technique of Folin,[15] modified only by the use of standard curves[19] in the interpretation of the colorimeter readings, the error, as we estimated it, amounts on the average to about 50 per cent of the quantity actually present. While therefore it is not possible to apply to each of the single determinations in our table a uniform correction, we are in a position to correct the average of any group of determinations in which the blood was not laked. Two such averages appear in Table I; one is that of 17 out of the 30 results in Group I, the other that of 27 (including the above 17) out of the total 60. The two averages are practically identical (1.57 and 1.58). If these are 50 per cent in excess of the true average, the latter will be found by deducting from each one-third. This gives a corrected average of 1.05 for both cases.

[26] Plass (*Bull. Johns Hopkins Hosp.*, 1917, xxviii, 137) states that the serum creatinine of women in parturition is the same as that of normal non-pregnant women, which again is somewhat lower than in normal men; but detailed data in support of these statements are not presented.

A comparison of this corrected average with the averages for the two corresponding groups of plasmas (1.09 and 1.03) shows that the difference in the creatinine content of whole blood and plasma is in reality practically negligible. It is, of course, not possible to say that there may not be in individual specimens a slight excess of creatinine in corpuscles or in plasma; but the conclusion to which the twenty-seven results considered indubitably appear to point is that in general the *creatinine of normal human blood is distributed among its different elements at a practically uniform concentration.* Our previously expressed opinion upon the point is therefore abandoned, and the contention of Wilson and Plass sustained.

Lacking quantitative data upon the average error of the Myers' technique, we are unable to apply any appropriate correction to results obtained upon blood which had been laked. Those figures, therefore, which are in parentheses in the table, cannot be utilized in the present argument. It will be noticed that the disproportion between them and the corresponding plasma results is in general greater than in the case of unlaked blood. Thus, the average for all 60 bloods, including as it does 33 which were laked, is not 50 but 75 per cent higher than the plasma average; and in individual cases, especially, it would seem, among puerperal women, laked blood appears to have twice or even thrice as much creatinine as its plasma. All this is in accord with our previous observations, and those of Wilson and Plass, upon the excessive tendency of the Myers' procedure to exaggerate the whole blood creatinine. It may be remarked, though, that this tendency is much less obvious in the strictly normal cases of Group I than in either of the other groups.

The conclusion that the creatinine content of blood is in reality the same as that of its plasma has an obvious bearing upon the question of the normal limits within which the whole blood creatinine may range. According to Folin and Denis[27] these are 1.0 to 1.4 mg. per 100 cc.; according to Myers and Fine,[28] 1.0 to 2.0. With such estimates as these our own uncorrected results with whole blood are in substantial agreement.

[27] Folin, O., and Denis, W., *J. Biol. Chem.*, 1914, xvii, 487.

[28] Myers and Fine, *Post-Graduate*, 1915, xxx, 39.

Our minimum is 1.1, our maximum 2.8; if results in puerperal women, which appear to be aberrant, are excluded, our maximum is reduced to 2.4, or, if we further limit the record to analyses of unlaked blood, to 2.1. Now, if it is granted that creatinine is uniformly distributed throughout the blood, the correct creatinine content of the latter will be given not by the figures just quoted, which we believe to exceed the truth, but by those for plasma, which we have shown[14] to be substantially free from error. On that basis the normal range of blood creatinine is found to be 0.70 to 1.3, with an average of practically 1 mg. This range is all but identical with that which may be found among results for human plasma reported by Plass[26] and by Wilson and Plass.[13] It indicates for the true creatinine content of blood a somewhat lower general level than the figures of Myers and Fine or even of Folin and Denis. It remains, nevertheless, of the same order of magnitude as these, and is very far from approaching the extraordinarily low estimate of Gettler and Baker.[29] These authors found that out of 30 bloods 26 contained only 0.1 mg. or less of creatinine per 100 cc.; and Gettler,[30] in a subsequent analysis of 11 other specimens, indicates 0.5 as the upper limit of normality, and reports only 5 as having more than 0.1. For such results as these we have failed to find either confirmation or explanation. The work of Gettler may be admitted to prove (see his Experiment V[30]) that the limits of 1.0 to 1.4, as found by Folin and Denis, should be corrected to 0.95 to 1.7; but that will hardly serve to explain how Gettler and Baker obtain values as low as 0.1.

Relation between Plasma and Urinary Creatinine.

The determination of urinary creatinine was undertaken simply as a step in the detection and estimation of creatine, and not with any deliberate intention of studying the laws regulating its own passage through the kidney. Any such purpose would have necessitated the taking of many precautions which for the present were neglected. It did, nevertheless, seem possible that even

[29] Gettler, A. O., and Baker, W., *J. Biol. Chem.*, 1916, xxv, 211.
[30] Gettler, A. O., *J. Biol. Chem.*, 1917, xxix, 47.

the haphazard procedure followed might reveal some rough relationship between the plasma and the urinary concentrations of creatinine. As far as comparison of the two in individual cases goes, no indication whatever of any such relationship can be detected. On the other hand, when regard is paid only to the various averages scattered through Table I, a certain degree of correspondence becomes apparent. Indeed, if one limits the attention to the averages in which the number of urines concerned is more than ten, the correspondence becomes a fairly close one. The averages in question are for plasma 0.87, 0.89, 0.99, 1.03, 1.04, 1.09, and for urine 95, 124, 141, 166, 157, 163. It seems probable, therefore, that the plasma concentration is at least one among the many factors upon which the concentration of creatinine in the urine must depend.

The Distribution of Creatine in Blood.

The greater number of our creatine determinations had been already carried out, before it became obvious, through the work of Wilson and Plass,[13] Greenwald,[31] and ourselves,[14] that the method employed, which in the beginning was the only one available, fell more than a little short of the accuracy necessary to our purpose. It seemed then best, instead of turning to other methods of as yet unproved reliability, to complete the series as it had been commenced, and to hope that, when due allowance had been made for the errors incurred, the results might prove to be not altogether without significance. The hope has been, we think, to a certain extent realized. At the same time we cannot, for conclusions based upon admittedly inaccurate data, claim more than a provisional validity. In the discussion which follows we wish therefore to be understood as dealing for the most part with suggestion rather than proof. Many, if not all, of the deductions drawn from our figures demand confirmation by a less dubious analytical method.

On one point perhaps the results do actually amount to a demonstration. In every case the plasma yields for creatine a figure so far below that of the blood, that there can hardly exist a doubt that the bulk of that substance is carried in the cor-

[31] Greenwald, I., *Proc. Soc. Exp. Biol. and Med.*, 1916–17, xiv, 115.

puscles. This conclusion, already formulated in our earlier contribution[12] to the subject, and later confirmed by Wilson and Plass,[13] is not affected by the respective errors of the creatine determinations in blood and in plasma; for these are of such relative magnitude that existing differences are more likely to be concealed by them than exaggerated. We have shown[14] reason to suspect that the true creatine content of the blood is, roughly speaking, about one-half, and that of the plasma about one-fourth, of the amount indicated in the colorimetric analysis. This estimate is not of such a precise character that it can be fairly applied to individual pairs of results, but it is perhaps not too crude to be used in a tentative correction of our averages. Modified accordingly, these averages vary, in the different groups distinguished, from 2.7 to 3.5 in the case of whole blood, and from 0.40 to 0.62 in the case of plasma. The corrected averages for all cases in which creatine was determined at all are 2.97 (practically 3.0) and 0.46. In general the proportion of whole blood to plasma creatine is about 6 or 7 to 1. Taking the average values reached along with the average plasma volumes corresponding, it is possible to calculate the average amount of creatine in the corpuscles; this appears to lie, in one group or another, between 6 and 9, with an average for all cases of 6.7 mg. per 100 cc.[32] It may be remarked that all the figures for creatine, and especially those for corpuscular creatine, are higher in the female groups than in the male. These quantitative data are offered, as we have said, with due reserve and as first approximations at the best.

[32] There are no available data with which our estimates of corpuscular creatine can be directly compared; but Plass (*Bull. Johns Hopkins Hosp.*, 1917, xxviii, 297), who employs an improved analytical method, has recently published figures ranging from 5.99 to 15.29 (average, 10.8) as the *total* corpuscular creatinine of ten parturient women. He states further, without recording details and without indicating clearly whether he is dealing here with creatine or total creatinine, that non-pregnant women yield much lower values (6.2 to 6.5), and that in one male the corpuscular concentration was lower still (4.9). Our figures, it will be seen, agree with those of Plass in indicating a smaller proportion of creatine in the corpuscles of the male, and a special accumulation in those of pregnant women. Even with regard to the absolute amounts present there exists a gratifying measure of agreement between results obtained in such different ways.

If they should be confirmed, it would follow, after what has been said of the equal distribution of creatinine, that the corpuscles contain from 5.5 to 10 times as much creatine as creatinine,[33] while in the plasma it is the latter which is predominant.

Relation between Creatine of Plasma and of Urine.

This leads us to consider whether our results throw any light upon the question of the relation between the concentration of creatine in the plasma and its occasional appearance in the urine. Assuming that the permeability of the healthy kidney cells to creatine is constant, there would appear to be only two forms which this relation might assume; either creatine is promptly excreted upon its earliest appearance in a plasma which is normally creatine-free, or else it passes the kidney only when its plasma concentration rises above a certain definite threshold.[34] Now, if the individual data for plasma and urinary creatine are compared, it is impossible to detect any uniform relation between them. Plasma creatines of from 1.4 to 3.2 seem to be associated indifferently with the presence of creatine in the urine or its absence. It can perhaps be said that whenever the (uncorrected) plasma creatine is lower than 1.4 the urine is creatine-free, and that whenever it is above 3.2 creatinuria makes its appearance; but the generalization includes in its scope such a small proportion of our cases that it possesses little significance. This apparent lack of a consistent relation is probably in part the consequence of the variable error involved in the single determination of plasma creatine. At any rate, when we compare averages, in which the error may be supposed to be more nearly constant, a certain regularity becomes at once apparent.

In order to exhibit this more clearly we have in the

[33] In our preliminary communication[12] (p. xix), in the sentence dealing with the relative amounts of creatinine and creatine in the corpuscles, the words "first" and "second" are transposed in such a manner as to convey a meaning exactly the opposite of what was intended. When the abstract was printed this unfortunately escaped detection.

[34] The dependence of creatinuria upon a specific increase of renal permeability constitutes a third possibility which cannot be entirely excluded, but for which there would not exist an exact analogy.

TABLE I.

No.	Case.	Age.	Sex.	Blood.					Urine.		Remarks.
				Creatinine.		Creatine as creatinine.		Relative plasma volume.	Creatinine.	Creatine as creatinine.	
				Whole blood.*	Plasma.	Whole blood.	Plasma.				
						Group I.					
				mg.	*mg.*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	
1	Y. B.....	25	M.	(1.8)	0.80	5.2	1.3	54	144	0	
2	P. R. S.....	31	"	1.2	0.84	6.8	2.0	60	95	5	
3	J. M.....	26	"	(1.5)	0.92	6.1	3.2	48	121	0	
4	A. B. H.....	24	"	1.6	0.92	5.7	1.9	63	182	7	
5	E. C. T.....	22	"	(1.9)	0.92	5.6	1.3	50	97	0	
6	C. A. Wa.....	25	"	1.4	0.93	5.3	1.9	61	61	2	
7	F. R. S.....	23	"	1.3	0.96	5.7	1.4	60	162	0	
8	R. C. C.....	24	"	1.3	0.97	5.8	1.3	58	259	0	
9	J. H. H.....	24	"	1.3	1.0	5.8	1.3	60	308	0	
10	G. C. M.....	30	"	1.6	1.0	6.0	1.4	61	60	3	
11	H. A. R.....	25	"	(1.8)	1.0	5.3	1.8	58	208	37	
12	R. C. M.....	23	"	(1.7)	1.1	4.2	1.3	54	97	0	
13	C. A. We.....	27	"	2.1	1.1	4.4	1.4	57	104	7	
14	R. H.....	24	"	(1.8)	1.1	7.0	2.2	59	231	0	
15	A. G.....	25	"	1.6	1.1	5.8	1.6	63	210	0	
16	A. H.....	40	"	(1.5)	1.1	4.7	1.4	62	88	0	
17	W. R. C.....	26	"	1.4	1.1	5.3	1.8	63	—	—	11 weeks later than Nos. 18 and 19.
18	"			(1.5)	1.1	4.3	1.1	63	144	0	
19	"			(1.6)	1.2	4.4	1.4	57			Same blood as No. 18, but hirudinized instead of oxalated.
20	A. H.....	40	"	(1.6)	1.2	4.6	1.6	57			Same blood as No. 16, but hirudinized instead of oxalated.

21	H. K. D.....	30	M.	1.8	1.2	5.5	1.6	57	308	0	
22	D. S. M.....	30	"	1.9	1.2	5.7	1.2	59	140	0	
23	F. M.....	33	"	1.8	1.2	5.6	1.4	58	164	0	
24	C. O. M.....	27	"	(1.5)	1.2	6.5	2.3	57	107	0	
25	W. A. S.....	32	"	(1.7)	1.2	5.3	2.5	56	95	0	
26	C. V. S.....	26	"	1.5	1.2	5.1	1.3	62	180	0	
27	T. S.....	27	"	(2.1)	1.2	7.3	2.3	58	172	0	
28	J. R. S.....	27	"	1.7	1.2	5.3	1.1	64	216	0	
29	J. Re.....	28	"	1.6	1.3	5.9	2.9	61	274	20	
30	A. H.....	40	"	1.8	1.3	—	—	61	—	—	13 weeks later than Nos. 16 and 20.
Average of Group I.....				(1.63)	1.09	5.52	1.70	59	163	3.1	
Same, corrected.....				—	1.09	2.76	0.42				Creatine of corpuscles = 6.1.
Average of laked bloods (13).....				(1.69)	1.08	5.42	1.82	57			
" " unlaked " (17).....				1.58	1.09	5.61	1.59	61			
Same, corrected.....				1.05	1.09	2.80	0.40				Creatine of corpuscles = 6.6.
Group II A.											
31	G. H.....	23	F.	(1.2)	0.72	6.1	2.0	61	124	14	Chronic constipation. In bed.
32	G. D.....	55	M.	(1.8)	0.76	4.8	1.0	54	74	0	Sprain. In bed.
33	W. D.....	35	"	(2.2)	0.80	6.9	1.7	63	132	0	Hernia, 10 days after operation. In bed.
34	W. Be.....	29	"	(1.8)	0.81	3.7	1.3	64	101	0	Undescended testicle, 7 days after operation. In bed.
35	W. T.....	48	"	(2.2)	0.83	5.2	1.6	59	46	11	Hydrocele, before operation. Ambulant.
36	J. C.....	26	"	(1.8)	0.91	6.9	1.0	46	247	0	Fracture. Ambulant.
37	E. F.....	21	"	(1.4)	0.91	7.4	1.3	54	84	2	Hernia, before operation. In bed.
38	P. V.....	12	"	(1.7)	0.92	4.5	1.4	66	129	8	Lacerated thumb. In bed.
39	G. M.....	25	"	(2.0)	0.98	4.2	1.4	55	152	4	Hernia, 7 days after operation. In bed.

* Determinations for the figures in parentheses were obtained by Myers' technique. Averages in which these were included are likewise in parentheses.

TABLE I—*Concluded.*

No.	Case.	Age.	Sex.	Blood.					Urine.		Remarks.
				Creatinine.		Creatine as creatinine.		Relative plasma volume.	Creatinine.	Creatine as creatinine.	
				Whole blood.*	Plasma.	Whole blood.	Plasma.				
				Group II B.							
				mg.	*mg.*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	
40	V. S.	18	F.	(1.7)	0.70	9.5	3.5	69	125	65	Puerperium, 9th day.
41	F. K.	23	"	1.5	0.78	7.2	1.9	67	53	5	Hysteria. In bed.
42	L. Le.	22	"	(2.6)	0.80	5.1	1.9	59	79	28	Puerperium, 8th day.
43	M. W.	35	"	1.5	0.86	7.3	2.1	67	43	12	Hysteria. In bed.
44	E. H.	19	"	(2.8)	0.87	4.8	2.0	57	99	39	Puerperium, 11th day.
45	E. T.	19	"	(2.3)	0.91	5.7	3.6	60	165	57	" 8th "
46	J. R. C.	39	M.	1.8	0.92	—	—	67	—	—	Nerve suture. In bed.
47	A. C.	40	"	1.5	0.95	6.8	2.1	61	—	—	Fracture. In bed.
48	J. B.	28	"	1.6	1.0	6.3	2.0	60	178	0	" Ambulant.
49	W. Br.	29	"	1.5	1.0	6.0	1.4	62	277	0	" "
50	F. D.	32	"	2.0	1.3	—	—	63	—	—	After hernia operation. Ambulant(?).
Average of Group II				(1.85)	0.89	6.02	1.84	61	124	14.4	
" " " " corrected				—	0.89	3.01	0.46				Creatine of corpuscles = 7.0.
" " males only				(1.79)	0.93	5.70	1.47	60	142	2.5	
" " females "				(1.94)	0.81	6.53	2.43	63	98	31.4	

Group III.

51	J. E.	36	F.	1.3	0.71	7.7	—	—	—	—	Ante partum, 8 days.
52	J. Ri.	18	"	(1.7)	0.72	5.5	1.8	61	95	17	" " 5 weeks.
53	B. S.	29	"	(1.7)	0.78	6.6	1.9	72	100	0	" " 19 days.
54	C. G.	28	"	(2.0)	0.82	5.5	2.4	59	67	—	" " 4 "
55	F. W.	37	"	1.8	0.89	7.9	—	—	96	34	" " 4 "
56	L. Li.	32	"	1.1	0.89	6.9	—	—	111	10	" " 2 "
57	B. C.	23	"	(1.7)	0.99	6.0	2.5	64	81	27	" " 1 day.
58	B. R.	28	"	(2.0)	1.1	9.6	2.3	71	—	—	" " a few hours.
59	E. R.	29	"	(2.1)	1.1	9.5	3.4	68	—	—	" " 11 days.
60	M. F.	25	"	(2.4)	1.2	4.8	3.0	70	—	—	" " 15 "
Average of Group III				(1.78)	0.92	7.00	2.47	66	92	17.6	
" " " " corrected				—	0.92	3.50	0.62				Creatine of corpuscles = 9.1.

General averages.

All cases (60)	(1.73)	0.99	5.94	1.85	60	141	8.6	
Same, corrected	—	0.99	2.97	0.46				Creatine of corpuscles = 6.7.
All males (43)	(1.68)	1.04	5.57	1.64	59	157	2.9	
All females (17)	(1.85)	0.87	6.81	2.45	65	95	25.7	
All with blood unlaked (27)	1.57	1.03	6.08	1.67	61	166	5.0	
Same, corrected	1.05	1.03	3.04	0.42				Creatine of corpuscles = 7.1.

* Determinations for the figures in parentheses were obtained by Myers' technique. Averages in which these were included are likewise in parentheses.

first part of Table II arranged all the averages for plasma creatine (none of which is drawn from less than seven separate determinations) in ascending order of magnitude, placing beside each of them the average urinary creatine of the group to which it belongs. The figures are then seen to fall readily into three sets. In the first, consisting of four examples with a plasma creatine (corrected) of about 0.4, the urinary creatine is never greater than 5; in the second, two examples with a plasma creatine of 0.46, the urinary creatine lies between 8 and 15; while in the third, three examples with a plasma creatine of about 0.6, the urinary creatine ranges from 17 to 32. Unfortunately the evidential value of this apparent correspondence is not so great as it seems; for, in consequence of the occasional incompleteness of the analytical record, the respective averages for plasma and for urine are not invariably drawn from the same set of individuals within the group which they are taken to represent. From this objection the figures which constitute the second part of Table II are free. They are averages which have been computed from those cases only in which *both* urine and plasma were successfully analyzed for creatine. The cases conforming to this requirement (46 in number) have been regrouped with reference simply to the concentration in which creatine was being excreted at the time of the analysis. Thus in the first group (26 cases) the urine was creatine-free; in the second (9 cases) it contained less than 10 mg. per 100 cc.; the third included all of the 20 cases in which any creatine at all was present; while the fourth embraced those only (11) in which the concentration of creatine was higher than 10 mg. per 100 cc. The data yielded by these groups indicate with clearness the association of a regular increase in the average creatine content of the urine with an equally regular, though of course much more gradual rise in the average level of the plasma creatine. They therefore afford an unequivocal confirmation of the conclusions to which the first part of the table had already pointed,—that the concentration of creatine in the urine bears a direct relation to that in the plasma, and that a slight increase in the latter (as little as 0.1 mg. per 100 cc.) suffices to bring about a copious excretion.

Had it been possible to put absolute confidence in the analytical data for plasma creatine, the last results would have demon-

TABLE II.

Comparison of Average Creatine Contents of Urine and Plasma.

Description of group.	No. of cases.	Plasma creatine per 100 cc.		Urinary creatine per 100 cc.
		Found.	Corrected.	
A. Grouping determined by case classification.				
		mg.	*mg.*	*mg.*
Males of Group II	11	1.47	0.37	2.5
All males	40	1.64	0.41	2.9
" with blood unlaked	21	1.67	0.42	5.0
Group I (males)	29	1.70	0.42	3.1
" II (males and females)	18	1.84	0.46	14.4
All cases	54	1.85	0.46	8.6
Females of Group II	7	2.43	0.61	31.4
All females	14	2.45	0.61	25.7
Group III (females)	7	2.47	0.61	17.6
B. Grouping determined by concentration of urinary creatine.				
Urines creatine-free	26	1.59	0.40	0
" containing less than 10 mg	9	1.62	0.41	4.8
" " any creatine whatever	20	2.02	0.51	18.5
" " more than 10 mg	11	2.34	0.59	29.7

strated one point more; the existence, namely, of a threshold for creatine, the level of which they would have fixed sharply at 0.4 mg. per 100 cc. But the unreliability of the determination renders exceedingly doubtful the significance of a quantity as small as that. Indeed the extreme lowness of the threshold indicated (it would be surprisingly low even if the original figures needed no correction) is enough in itself to throw suspicion upon its reality. Thresholds of established importance, like those of glucose and chlorides, seem to be generally of much greater magnitude. The idea that there may be a normal constant of this sort for creatine receives moreover, it must be admitted, no support from the results of Wilson and Plass,[13] who used a method of analysis presumably more accurate than ours. The figures they present indicate rather the complete absence of creatine from the plasma in cases where the urine is ordinarily creatine-free, and its presence in those only where creatinuria

is the rule. Their evidence, unfortunately, is in some respects as defective as our own. The number of human cases they report is but nine in all, and in none of them was the urine actually analyzed. A final settlement of the question of a creatine threshold must therefore await the collection, by methods of unquestioned accuracy, of data more complete and reliable than any that we yet possess.

SUMMARY.

1. The creatinine content of normal human blood plasma ranges under different conditions from 0.70 to 1.3 mg. per 100 cc., the average for 60 specimens examined being 1 mg.

2. It is practically certain that the creatinine of normal blood is distributed through corpuscles and plasma in uniform concentration.

3. The figures given for plasma indicate, therefore, also the true creatinine content of whole blood.

4. The blood creatinine is apt to be lower in females than in males, and lower in subjects deprived of exercise than in those leading an active life. It is suggested that the blood creatinine is related to muscular development in much the same way as the creatinine coefficient.

5. The creatine of the blood is chiefly concentrated in the corpuscles. With the method used exact determinations were unattainable, but it is roughly estimated that the average creatine content of the corpuscles lies between 6 and 9 mg. per 100 cc., while that of the plasma is not more than 0.4 to 0.6. The blood as a whole contains apparently an average of about 3 mg. per 100 cc. There seems to be more in the blood of females than of males.

6. There is a distinct correspondence between increase of plasma creatine and the appearance of creatine in the urine; but whether the plasma, in the absence of creatinuria, is creatine-free or whether there exists a threshold for creatine excretion, has not been positively determined. If there is a threshold, it is a very low one.

We take this opportunity of thanking the students and instructors of Toronto University Medical School who permitted us to

examine specimens of their blood. It is a pleasure also to acknowledge the courtesy with which Doctors B. P. Watson, K. C. McIlraith, A. McPhedran, G. W. Ross, W. B. Thistle, F. N. G. Starr, and G. A. Bingham, all of the staff of the Toronto General Hospital, permitted us to use material from the wards under their charge.

THE INFLUENCE OF THYROID FEEDING UPON CARBOHYDRATE METABOLISM. I.

THE STORAGE AND MOBILIZATION OF THE LIVER GLYCOGEN IN THYROID-FED ANIMALS.

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(Received for publication, November 24, 1917.)

An earlier paper[1] dealt chiefly with the influence of thyroid feeding upon the glycogen content of the liver and the sugar content of the blood. In the present series, I have investigated the glycogenetic and glycogenolytic action of the liver of thyroid-fed animals, the influence of thyroid feeding upon the liver glycogen of frogs, the interrelations of the thyroid and adrenals, acidosis after thyroid feeding, and the influence of alkali upon the diminished content of liver glycogen of thyroid-fed animals. Desiccated thyroid (Parke, Davis and Company) was usually fed, although fresh pig thyroid was used in one series of experiments. The methods employed for feeding and for determining the liver glycogen and the sugar of the blood or urine were the same as described in the first paper,[1] unless otherwise specified.

The Formation of Liver Glycogen in Thyroid-Fed Rats upon a Very High Carbohydrate Diet.

The glycogen content of the liver is rapidly decreased to the minimum by thyroid feeding, and is readily restored by omitting thyroid tissue from the diet. On the other hand, the liver of the thyroid-fed animals apparently does not increase its glycogen content as easily as that of fasted rats after parenteral administration of dextrose.[1] The increased need for energy-producing materials in hyperthyroidism may be the only cause of these phenomena, or it may also be that the glycogenetic or glycogenolytic action of the liver is somewhat affected. Though *in vivo* the

[1] Kuriyama, S., *Am. J. Physiol.*, 1917, xliii, 481.

thyroid hormone is thought to accelerate the oxidation of carbohydrates,[2] King[3] reported that *in vitro* the dextrose-destroying power of a mixture of muscle and pancreatic juice (reported as polymerization by others) is markedly retarded by the addition of thyroid juice. Mackenzie,[4] however, observed that thyroid juice has no influence upon blood glycolysis *in vitro*. Opinions as to the glycogenetic function of the liver in some pathological conditions, where the liver glycogen promptly disappears, are still controversial. Grube[5] claims that perfusion of livers with phlorhizin not only prevents formation of glycogen from dextrose, but also breaks down part of the glycogen previously existing. Schöndorff and Suckrow,[6] repeating these experiments, failed to confirm them. According to Neubauer,[7] in phosphorus poisoning as well as in pancreatic diabetes, the liver is affected in its function of forming glycogen from glucose, while this does not hold for levulose. Nishi's experiments,[8] however, show that the glycogenetic function of the liver of depancreatized animals remains normal.

If an increased oxidation is the only or at least the chief cause of the decrease of liver glycogen in hyperthyroidism, a large amount of a very high carbohydrate diet might cause the storage of glycogen in the liver of thyroid-fed animals. Some experiments were made to test this.

Methods.—As the usual food, a paste, made of 70 parts of dog biscuit (rat biscuit which is specially prepared for feeding experiments by Drs. Osborne and Mendel was not used this time) and 30 parts of lard, was given, and as extra carbohydrate, sucrose was added. Giving the dog biscuit-lard diet freely to normal rats, Osborne and Mendel[9] reported that about 4.5 to 9 gm. per day of the food administered to a 150 gm. albino rat or 9 to 13.5 gm. to a 300 gm. rat, are enough to maintain or even to increase the body weight. In my experiments, owing to the frequent decrease of the appetite of the thyroid-fed animals, not much more food than this average could be given. 1 gm. of

[2] Cramer, W., and McCall, R., *Quart. J. Exp. Physiol.*, 1917, xi, 59.

[3] King, J. H., *J. Exp. Med.*, 1909, xi, 665.

[4] Mackenzie, G. M., *J. Exp. Med.*, 1915, xxii, 757.

[5] Grube, K., *Arch. ges. Physiol.*, 1909, cxxviii, 118.

[6] Schöndorff, B., and Suckrow, F., *Arch. ges. Physiol.*, 1911, cxxxviii, 538.

[7] Neubauer, E., *Arch. exp. Path. u. Pharm.*, 1909, lxi, 174.

[8] Nishi, M., *Arch. exp. Path. u. Pharm.*, 1910, lxii, 170.

[9] Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington. Publication No. 156*, pt. i, 1911, 16.

desiccated thyroid, mixed with 2 gm. of paste, was given in the afternoon, and after this food was eaten, 10 gm. of paste were put in the cage. On the last day of the experiment, 1 gm. of paste with desiccated thyroid was given at 9 p.m. After this was eaten, 1 gm. of paste and a certain amount of sucrose, mixed with a small amount of water, were given. At 9 the next morning the animal was killed and the liver glycogen determined. As control experiments, a few rats were fasted for 72 hours. At the end of this period, a certain amount of paste or sucrose was given to some of them, the rest still kept fasting. 12 hours later all the animals were killed and the liver glycogen was determined. Neither thyroid-fed, nor control animals showed any diarrhea. The results are detailed in Tables I and II. Rats III, IV, and V in Table II are the same animals which were shown in this *Journal*.[10] The body weight of these three was taken at the end of the 72 hour fasting. In calculating the calories of the food of the last experimental day, the dog biscuit-lard mixture, designated as "paste," was considered to have 5 calories per gm., desiccated thyroid 4.1 calories per gm.

As will be seen in these tables, the storage of glycogen in the liver of thyroid-fed rats is much less than in fasted rats. In some thyroid-fed rats, however, the liver was able to store a noteworthy amount of glycogen. A 72 hour fast decreases the liver glycogen to the minimum; and a small amount of paste or sucrose was enough to make it reappear abundantly within 12 hours. During the same length of time, the thyroid-fed rats ingested several times as many calories as the fasted animals.

Rudinger[11] reported that in Graves' disease the oxidation of protein is abnormally increased and the administration of too much protein accelerates the activity of the thyroid, while the diet with a large amount of carbohydrate has a counter effect, the hyperfunction of the thyroid being retarded by an antagonistic action of the accelerated pancreatic function. According to Cramer and McCall,[2] the oxidation of carbohydrate is also increased in hyperthyroidism. Investigating the respiratory gas

[10] Kuriyama, S., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 136.
[11] Rudinger, K., *Wien. klin. Woch.*, 1908, xxi, 1581.

TABLE I.

The Formation of Liver Glycogen in Thyroid-Fed Rats upon a Very High Carbohydrate Diet.

Rat			I	II	III	IV	V	VI	VII	VIII
Body weight, *gm*			226.5	306.1	215.7	298.9	285.9	273.1	288.5	210.9
Food: Desiccated thyroid, 1 gm., +	1st day.	Paste, *gm*	8.0	6.9	8.0	6.5	8.5	8.9	6.4	8.9
	2nd day.	Paste, *gm*	8.5	6.1	6.5	8.0	6.0	8.6	8.7	6.9
	3rd day.	Paste, *gm*	5.3	7.3	3.9	6.9	7.6	6.7	5.6	8.0
	4th day.	Paste, *gm*	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
		Sucrose, *gm*	3.0	3.0	3.0	8.0	7.0	5.0	6.0	5.0
		Calories	26.4	26.4	26.4	46.9	42.8	34.6	38.7	34.6
Loss of body weight, *gm*			−18.8	−13.2	−12.5	−22.5	−31.7	−29.1	−20.7	−21.0
Liver.	Weight, *gm*		6.8	9.1	5.8	11.0	7.3	6.7	11.1	7.4
	Glycogen content.	*mg*	0	50.0	Trace.	281.1	11.8	36.4	242.8	7.5
		per cent		0.6		2.6	0.2	0.5	2.2	0.1

interchange, Magnus-Levy,[12] Thiele and Nehring,[13] and Salomon[14] reported that both healthy men and myxedema patients treated with thyroid substance and also patients with Graves' disease show an increase of 20 to 80 per cent in the amount of oxygen consumed. In experimental hyperthyroidism under the present conditions this increase may be much higher. Although no diarrhea was evident in my experiments, digestibility of a very high carbohydrate diet must also be taken into consideration. The oft reported decrease of the assimilation limit of carbohydrate

TABLE II.

The Formation of Liver Glycogen by Administration of a Certain Amount of Food in Fasted Rats.

Rat			I	II	III	IV	V	VI	VII
Body weight, *gm*.			204.0	224.0	150.0	255.0	250.0	207.0	219.5
Food after 72 hour fasting period.	Paste, *gm*.		2.0	0.5	0	0	0	0	0
	Sucrose, *gm*.		0	1.5	2.0	5.0			
	Calories		10.0	8.7	8.2	20.5	0	0	0
Loss of body weight, *gm*.			−19.8	−25.7	−30.0	−38.0	−48.0	−24.5	−22.0
Liver.	Weight, *gm*.		6.0	6.2	4.2	6.9	4.5	5.3	4.9
	Glycogen content.	*mg*.	189.0	322.4	216.1	399.6	Trace.	0	Trace.
		per cent	3.2	5.2	5.2	5.8			

in Graves' disease does not seem to be so great and is not always an accompanying symptom. From my experiments, it will be seen that under suitable conditions, the liver of thyroid-fed rats can store a certain amount of glycogen. Whether the remarkable difference between the thyroid-fed and fasted animals in forming the liver glycogen is due only to an increase of general metabolism cannot yet be clearly answered.

[12] Magnus-Levy, A., *Berl. klin. Woch.*, 1895, xxii, 650; *Deutsch. med. Woch.*, 1896, xxii, 491.

[13] Thiele, O., and Nehring, O., *Z. klin. Med.*, 1896, xxx, 41.

[14] Salomon, H., *Berl. klin. Woch.*, 1904, xli, 635.

The Diastase Content of the Liver and Blood Serum of Thyroid-Fed Animals.

Opinions concerning the function of diastase in various animal fluids or tissues and alterations of its concentration in certain pathological conditions are quite controversial. Studying the behavior of the liver diastase in diabetes, Bang[15] reported that the glycogen-free liver of depancreatized dogs showed no excessive diastatic action. He considers this fact as an indication that diabetes depends not on excessive destruction but on deficient formation of glycogen. In the same type of experiments, Zegla[16] found a slight decrease of the liver diastase; but Hinselmann,[17] on the contrary, found an increase. Though Zegla[16] reported that the diastase content of the liver increases in phlorhizin or phloretin glycosuria, the slight difference in the results of his experiments seems still to be within the technical error or physiological variation. Wohlgemuth and Benzur[18] reported that phlorhizin, phloretin, or epinephrine injection causes an increase of the diastase content of the kidneys, while the diastase content of the liver and blood serum shows no decided change. Wohlgemuth[19] demonstrated also that fasting, change of diet, increase of pancreatic activity by hydrochloric acid or secretin have no effect upon the diastase content of the blood. Knowing that epinephrine, *piqûre*, and other nervous influences are without action upon the blood diastase, Moeckel and Rost[20] are of the opinion that the blood diastase is a product of the metabolism of the cells, which has no special significance and serves no function in the organism. Macleod and Pearce[21] reported that in an etherized animal after death there is usually, though not always, an acceleration in the rate of glycogenolysis of the liver. Studying the glycogenolytic power of the liver after splanchnic stimulation, the same authors[22] concluded that modifications in the glycogenolytic activity of the liver do not depend on changes in the amount of diastase, but on changes in the conditions under which a constant amount of this ferment is acting. Scaffidi[23] demonstrated that the liver of frogs poisoned with phosphorus has a glycogenolytic power as high as the normal liver. Bang[24] asserts that the liver of *Rana esculenta* contains a large amount of

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[15] Bang, I., *Beitr. chem. Physiol. u. Path.*, 1907, x, 320.

[16] Zegla, P., *Biochem. Z.*, 1909, xvi, 111.

[17] Hinselmann, H., *Z. physiol. Chem.*, 1909, lxi, 265.

[18] Wohlgemuth, J., and Benzur, J., *Biochem. Z.*, 1909, xxi, 460.

[19] Wohlgemuth, J., *Biochem. Z.*, 1909, xxi, 381.

[20] Moeckel, K., and Rost, F., *Z. physiol. Chem.*, 1910, lxvii, 433.

[21] Macleod, J. J. R., and Pearce, R. G., *Am. J. Physiol.*, 1910–11, xxvii, 341.

[22] MacLeod and Pearce, *Am. J. Physiol.*, 1911, xxviii, 403.

[23] Scaffidi, V., *Biochem. Z.*, 1915, lxviii, 320.

[24] Bang, *Biochem. Z.*, 1913, xlix, 40.

latent diastase, which does not act in the physiological formation of sugar in the liver, while the liver of *Rana fusca* contains only a small amount of such latent diastase. In human diabetes, Wynhausen[25] failed to demonstrate any decided change of the blood diastase, while Myers and Killian's recent studies[26] showed a noteworthy increase.

Feeding thyroid to cats, Burge, Kennedy, and Neill[27] found that the catalase content of the blood increases markedly. On the other hand, Juschtschenko[28] reported that thyroidectomy causes a decrease of the catalase content of the blood. Wells[29] sought for a possible influence on autolysis of the liver by thyroid extract, but could demonstrate none *in vitro*. Schryver,[30] however, found that autolysis was more rapid in the liver of dogs fed on thyroid extract for some days before death than it was in control animals.

Concerning the influence of the thyroid upon the blood diastase, Wohlgemuth[19] found a noteworthy decrease of the blood diastase in thyroidectomized goats. The diastase content of the blood serum of these animals was $D_{24\,h}^{38°} = 3.3 - 5$, while the normal goat serum was valued at $D_{24\,h}^{38°} = 20 - 40$.

I have made some experiments in order to ascertain whether the decrease of the glycogen content of the liver in hyperthyroidism has any relation to the glycogenolytic action of the liver and blood serum.

Records of these follow.

Methods.—Rabbits were used for blood diastase determination; rats for liver diastase. To the former, fresh pig thyroid was given; to the latter, desiccated thyroid. In rabbits, the blood was taken from an ear vein by puncture, and the serum separated. For determining the liver diastase, the organ was extirpated promptly after bleeding the animals and immersed in warmed physiological saline solution. After weighing, the liver was freed from blood by perfusing with the same saline solution. The liver was then thoroughly ground in a mortar with four times its weight of physiological saline solution and a small amount of sand, placed in an ice box for 1 hour, and shaken from time to time. After filtration through a cloth, the homogeneous organ

[25] Wynhausen, O. J., *Berl. klin. Woch.*, 1910, xlvii, 1281.

[26] Myers, V. C., and Killian, J. A., *J. Biol. Chem.*, 1917, xxix, 179.

[27] Burge, W. E., Kennedy, J., and Neill, A. J., *Am. J. Physiol.*, 1917, xliii, 433.

[28] Juschtschenko, A., *Biochem. Z.*, 1910, xxv, 49.

[29] Wells, H. G., *Am. J. Physiol.*, 1904, xi, 351.

[30] Schryver, S. B., *J. Physiol.*, 1904–05, xxxii, 159.

emulsion was used for diastase determination by Wohlgemuth's method.[18, 31] The results are shown in Tables III and IV.

TABLE III.

The Diastase Content of the Blood Serum of Thyroid-Fed Rabbits.

Rabbit	I	II	III*	IV†
Body weight, *kg*	3.30	2.64	2.86	2.58
Food	Oats.	Oats.	Oats + greens.	Oats + greens.
Fresh pig thyroid, 15 gm. per day	June 8–11	June 13–16	June 8–11	June 8–11
Diastase content of serum. $D^{37°}_{24h}$ =	62.5 May 25	62.5 June 12	62.5 May 24	62.5 May 24
	62.5 June 10	62.5 " 15	62.5 " 25	62.5 June 10
	62.5 " 11	62.5 " 15	62.5 June 11	100.0 " 11
	62.5 " 12	62.5 " 16	100.0 " 12	62.5 " 12

* Died on June 14. † Died on June 12.

TABLE IV.

The Diastase Content of the Liver of Thyroid-Fed Rats.

Thyroid-Fed Rats. Food, Desiccated Thyroid, 1 Gm. per Day; Paste, 4 Gm. per Day; Dog Biscuit, Freely; for 4 Days.

Rat		I	II	III	IV	V
Body weight, *gm*		128.1	162.9	127.2	109.0	133.0
Liver.	Weight, *gm*	7.4	9.3	6.4	4.6	5.0
	Diastase content, $D^{45°}_{24h}$ =	156	250	250	156	250

Control Rats. Food, Paste, 5 Gm. per Day; Dog Biscuit, Freely; for 4 Days.

Rat		I	II	III	IV
Body weight, *gm*		117.3	148.4	219.0	301.4
Liver.	Weight, *gm*	4.4	6.1	9.4	10.3
	Diastase content, $D^{39°}_{24h}$ =	156	156	250	250

[31] Wohlgemuth, *Biochem. Z.*, 1908, ix, 1.

As the diastase content of the blood serum of normal rabbits, Wohlgemuth and Benzur[18] obtained the value of $D_{24}^{35°}h = 62.5$, which sometimes increased to 100 or 150. In my normal rabbits, the blood serum always showed the value of $D_{24}^{35°}h = 62.5$. After thyroid feeding, the diastase content of the blood serum remained about the same. As to the diastase content of the liver, the animal species seem to show some noteworthy difference. In rabbits it is said to vary between $D_{24}^{35°}h = 5$ and 12.5 (Wohlgemuth and Benzur[18]), while in rats $D_{24}^{35°}h$ is as high as 80 (meat feeding) or 160 to 320 (bread or lard feeding) (Hirata[32]). In my own experiments, the diastase content of the normal rat liver was $D_{24}^{35°}h = 156$ to 250. This accords well with Hirata's results. In thyroid-fed rats, the value was about the same. As far as the diastase content of the blood serum and the liver is concerned, the decrease of the glycogen content of the liver in experimental hyperthyroidism does not seem to be due to an increased glycogenolytic power of the liver or blood.

The diastase content, however, is always determined on the dead material. The diastatic power of such substances may not be identical with that in the living condition. Comparing the diastatic efficiency and average glycogen content in the different tissues and organs, MacLean[33] reported that the diastatic power and the glycogen content do not necessarily run parallel and sometimes an organ containing merely a trace of glycogen, *e.g.*, the lung, shows a more marked amylolytic power than a glycogen-rich organ, such as the liver. Moreover, a long list of substances, which accelerate or inhibit the activity of diastase, has been reported. A small amount of such substances within the living cells may markedly affect the activity of this enzyme. It is a well known fact that a marked increase of protein and fat metabolism exists in hyperthyroidism. The nitrogenous constituents of the urine are usually increased. Bock[34] reported an increase of amino-acid nitrogen of the blood in a case of hyperthyroidism (9.58 mg. amino-acid N in 100 cc. of blood; the nor-

[32] Hirata, G., *Biochem. Z.*, 1910, xxvii, 385.
[33] MacLean, H., *Biochem, J.*, 1909, iv, 467.
[34] Bock, J. C., *J. Biol. Chem.*, 1917, xxix, 191.

mal blood containing only 7.13 mg.). Many cleavage products of protein, such as amino-acids, creatine, creatinine, and others, have been reported to accelerate the activity of the diastase.[35] Though the liver and blood of thyroid-fed animals do not show any increased diastase content, it may be possible that some substances, which are produced to an abnormally large degree, on account of an increase of metabolism, accelerate the activity of diastase within the living cells.

The Influence of Thyroid Feeding upon the Glycogen Content of the Liver of Full Grown Frogs.

Determining the glycogen content of the frog (*Rana fusca*) at different seasons, Athansiu[36] reported that the glycogen content of the frog body is lowest in the summer, at which time they take the most nourishment, and the highest in the fall, gradually decreasing in the winter. At the end of hibernation the body still contains a large amount of glycogen. This statement was confirmed in general by Pflüger.[37] In frogs, glycosuria can be induced by subcutaneous application of phlorhizin, but not by oral administration.[38] Scaffidi[23] reported that phosphorus poisoning induces a marked loss of the liver glycogen of frogs (*Rana esculenta*) in a short time. Mangold,[39] however, observed that after the long continued convulsions caused by strychnine, which promptly reduce the liver glycogen of warm-blooded animals, the liver of a frog still contained a large amount of glycogen. As shown by Gudernatsch,[40] Lenhart,[41] and Graham,[42] thyroid feeding exerts a remarkable influence upon the growth of tadpoles. Emaciation, retardation in gain of body weight, and early differentiation of body parts can be noticed in a few days. Lenhart considers that the effect of thyroid feeding is closely associated with both the iodine content of thyroid and the amount fed.

In full grown frogs, the metabolism is probably most active in the summer time. It may be interesting to know whether in this season thyroid feeding of full grown frogs can increase the metabolism or at least cause any decrease of the glycogen con-

[35] Effront, J., *Compt. rend. Soc. biol.*, 1904, lvii, 234. Terroine, E. F., and Weill, J., *J. physiol. et path. gen.*, 1912, xiv, 437.
[36] Athansiu, J., *Arch. ges. Physiol.*, 1899, lxxiv, 561.
[37] Pflüger, E., *Arch. ges. Physiol.*, 1907, cxx, 253.
[38] Lusk, G., *Ergebn. Physiol.*, 1912, xii, 315.
[39] Mangold, E., *Arch. ges. Physiol.*, 1907–08, cxxi, 309.
[40] Gudernatsch, J. F., *Am. J. Physiol.*, 1914–15, xxxvi, 370.
[41] Lenhart, C. H., *J. Exp. Med.*, 1915, xxii, 739.
[42] Graham, A., *J. Exp. Med.*, 1916, xxiv, 345.

TABLE V.

The Glycogen Content of the Liver of Thyroid-Fed Frogs. Desiccated Thyroid, 1 Gm. per Day.

Frog		I	II	III	IV	V
Body weight, *gm.*		214.7	183.3	234.3	144.3	133.8
Duration of thyroid feeding, *days*		3	6	8	6	6
Change of body weight, *gm*		−3.6	+2.2	−9.0	+7.1	−3.2
Liver.	Weight, *gm*	4.5	6.1	5.3	4.1	3.6
	Glycogen content. *mg*	156.3	372.8	229.0	215.5	175.5
	Glycogen content. *per cent*	3.5	6.1	4.3	5.3	4.9

Control Frogs.

Frog		I	II	III	IV
Body weight, *gm*		195.9	133.3	195.6	136.9
Food		No food for 3 days.	No food for 6 days.	Powdered egg, 1 gm. per day, for 8 days.	Powdered egg, 1 gm. per day, for 6 days.
Change of body weight, *gm*		−1.8	0	+2.6	+7.8
Liver.	Weight, *gm*	4.5	3.2	3.9	3.9
	Glycogen content. *mg*	274.0	117.2	104.0	151.2
	Glycogen content. *per cent*	6.1	3.7	2.7	3.9

tent of the liver. Some experiments bearing upon this point are recorded below.

Method.—The experiments were carried on with *Rana pipiens* in June. Desiccated thyroid, made into a paste by mixing with a small amount of water, was given orally. The dose of desiccated thyroid was 0.5 gm., twice daily. The animals were kept in running tap water and no food was given to them. The control frogs were divided into two groups. One group was kept in running water only. To the other group, boiled, desiccated, and powdered egg was fed, in quantities comparable to those of the thyroid given. The results are detailed in Table V.

As shown in this table, there is no decided difference in the glycogen content of the livers of thyroid-fed frogs and those of control animals. The glycogen content of the liver of thyroid-fed frogs was 4.8 per cent (the average of 5 cases), while in the control frogs it was 4.1 per cent (the average of 4 cases). In none of the animals was any noteworthy change of the general conditions observed. Under the experimental conditions employed, thyroid feeding does not decrease the glycogen content of the liver. This is in harmony with the result obtained in a frog poisoned with strychnine and seems to indicate a great difference in the intensity of metabolism in the different stages of the life of the frog.

SUMMARY.

The storage of glycogen in the liver of thyroid-fed rats does not occur readily, even with the administration of a large amount of a very high carbohydrate diet. In fasted rats the liver glycogen reappears abundantly after ingestion of a comparatively small amount of food. If a sufficiently large calorific food intake is administered to thyroid-fed rats, liver glycogen may sometimes reappear to a limited extent. The quantity of glycogen so stored is, however, much smaller than that in fasted rats, subsequently given food with a fuel value several times less than that of the thyroid-fed rats.

The diastase content ($D_{24}^{37.5}h$) of the normal rabbit serum and the normal rat liver is about 62.5 and 156 to 250 respectively. Thyroid feeding does not change these values markedly.

The glycogen of the liver of thyroid-fed frogs (*Rana pipiens*) is practically the same as that of control animals. This accords with the results obtained with frogs poisoned with strychnine or in hibernation and perhaps indicates a notable difference in the intensity of metabolism of warm- and cold-blooded animals and of the frog at different life stages.

THE INFLUENCE OF THYROID FEEDING UPON CARBOHYDRATE METABOLISM. II.

THE EPINEPHRINE CONTENT OF THE ADRENALS OF THYROID-FED RATS.

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In my previous investigations,[1] the epinephrine content of the adrenals of white rats, fed on thyroid for a few days, was little different from that of control animals. A colorimetric method of estimation with mercuric acetate was used. Folin, Cannon, and Denis[2] reported that phosphotungstic acid (uric acid reagent) is a very sensitive reagent, solutions containing one part of epinephrine in 3,000,000 parts of water producing an unmistakable reaction with this reagent, and therefore being approximately ten times as sensitive as other chemical tests. Using Folin's method, Herring[3] reported that the administration of raw thyroid to cats and rats can increase the epinephrine content of the adrenals. The adrenals were also heavier than those of control animals. Hoskins[4] studied the influence of thyroid feeding upon the adrenal glands. In new-born guinea pigs, the adrenal glands were 25 per cent heavier than those of control animals. In young white rats this phenomenon was also confirmed. The heart, spleen, liver, and kidneys were also heavier than those of control animals.

The present experiments extend previous observations with the white rats.

Method.

Albino rats were divided into three series, each having nearly the same number of each sex. The body weight of each series averaged about the same. All of them were fed on dog biscuit-

[1] Kuriyama, S., *Am. J. Physiol.*, 1917, xliii, 481.

[2] Folin, O., Cannon, W. B., and Denis W., *J. Biol. Chem.*, 1912–13, xiii, 477.

[3] Herring, P. T., *Quart. J. Exp. Physiol.*, 1915–16, ix, 391; 1917, xi, 47.

[4] Hoskins, R. G., *J. Am. Med. Assn.*, 1910, lv, 1724. Hoskins, E. R., *J. Exp. Zool.*, 1916, xxi, 295.

lard diet. Two series were used for thyroid feeding, the third one serving as control. Each rat was kept in a separate cage. As thyroid administration 0.1 gm. of desiccated thyroid per day was prescribed to each rat of the one series, and this was continued for 23 days. Every rat of the other series received 1 gm. of desiccated thyroid per day for 4 days. Thyroid powder, mixed with two or three times the weight of the dog biscuit-lard paste, was readily eaten. After this had been consumed, the paste was given freely. At the end of the period, the animal was bled and both adrenals were carefully freed from the surrounding tissues and weighed together. The epinephrine content was determined by Folin's method, modified for smaller quantities of material. The protein of the ground adrenals was precipitated by acid and salt in a 10 cc. volumetric flask. The mixture was diluted up to the mark and filtered. 8 cc. of the filtrate were taken into a 25 cc. volumetric flask and the color, produced in this flask, was compared with the standard color in another 25 cc. flask. For the standard color, two 25 cc. volumetric flasks containing 0.2 and 0.4 mg. of uric acid, respectively, were provided and the flask which was the nearer in color to the epinephrine flask was used. The results are shown in Tables I and II.

TABLE I.

The Epinephrine Content of the Adrenals of Normal Rats.

Rat.	Sex.	Body weight.	Weight of two adrenals.	Weight of two adrenals per 100 gm. of body weight.	Epinephrine in two adrenals.	Epinephrine per gm. of adrenal.	Epinephrine per 100 gm. of body weight.
		gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
I	M.	304.8	36.9	12.1	0.102	2.77	0.033
II	"	269.7	35.3	13.1	0.094	2.66	0.035
III	"	254.3	63.1	24.8	0.162	2.57	0.064
IV	"	218.1	57.5	26.4	0.099	1.72	0.045
V	"	180.0	33.8	18.8	0.091	2.69	0.051
VI	F.	243.8	50.6	20.8	0.134	2.65	0.055
VII	"	240.2	50.6	21.1	0.174	3.44	0.072
VIII	"	199.4	39.5	19.8	0.098	2.48	0.049
IX	"	196.2	39.5	20.1	0.104	2.63	0.053
X	"	194.6	39.1	20.1	0.100	2.56	0.051
XI	"	69.2	25.4	36.7	0.056	2.20	0.081
Average.....		**215.5**	**42.8**	**21.3**	**0.110**	**2.58**	**0.054**

TABLE II.

The Epinephrine Content of the Adrenals of Thyroid-Fed Rats.

Rat.	Sex.	Change of body weight during experiment.	Body weight at end of experiment.	Weight of two adrenals.	Weight of two adrenals per 100 gm. of body weight.	Epinephrine in two adrenals.	Epinephrine per gm. of adrenal.	Epinephrine per 100 gm. of body weight.
Series A. Desiccated thyroid; 1 gm. per day for 4 days.								
			gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
I	M.	−17.2	276.4	42.0	15.2	0.095	2.26	0.034
II	"	−18.5	254.2	34.1	13.4	0.092	2.70	0.036
III	"	−23.0	244.0	34.8	14.3	0.093	2.67	0.038
IV	"	−14.9	126.9	28.1	22.1	0.059	2.10	0.047
V	"	−22.9	125.4	30.5	24.3	0.057	1.87	0.045
VI	F.	−17.0	267.8	44.9	16.8	0.092	2.05	0.034
VII	"	−18.8	189.9	48.3	25.4	0.106	2.20	0.056
VIII	"	−17.3	182.8	43.4	23.7	0.111	2.56	0.061
IX	"	−19.1	178.6	64.5	36.1	0.119	1.85	0.067
X	"	−18.8	178.1	43.4	24.4	0.084	1.94	0.047
XI	"	−17.8	173.4	44.3	25.5	0.101	2.28	0.058
Average.....			**199.8**	**41.7**	**21.9**	**0.092**	**2.23**	**0.048**
Series B. Desiccated thyroid; 0.1 gm. per day for 23 days.								
I	M.	−2.8	267.3	50.0	18.7	0.101	2.02	0.038
II	"	−5.4	231.6	41.5	17.9	0.098	2.36	0.042
III	"	−10.0	221.0	46.6	21.1	0.126	2.70	0.057
IV	"	−2.9	210.7	36.2	17.2	0.071	1.96	0.034
V	F.	−4.8	198.4	50.6	25.5	0.142	2.81	0.072
VI	"	+0.9	176.4	38.4	21.8	0.082	2.14	0.047
VII	"	−2.6	149.4	37.8	25.3	0.083	2.20	0.056
VIII	"	−14.1	144.6	42.1	29.1	0.081	1.92	0.056
Average.....			**199.9**	**42.9**	**22.1**	**0.098**	**2.26**	**0.050**

As the epinephrine content of the adrenals of normal animals (mg. of epinephrine per gm. of the gland), Folin and his coworkers[2] found 2.41 to 3.08 mg. in the sheep, 2.04 to 2.33 mg. in the dog, 3.33 to 3.52 mg. in the calf, 1.22 to 1.52 mg. in the young cat, and Herring found 1.25 to 2.22 mg. in the cat, and 1.47 to 2.97 mg. in the albino rat. In my experiments this value in albino rats averaged 2.20 mg. The maximum was 3.44

mg., the minimum 1.72 mg. It will be seen that the epinephrine content of the adrenal shows some variation according to both the animal species and the individual.

As the thyroid hormone is considered to stimulate the sympathetic nervous system, an excess of it in the body may stimulate the activity of the adrenal. But this may not necessarily be accompanied by a higher content of the epinephrine in the gland. The excess of epinephrine may immediately be transported into the circulation, its concentration in the gland being kept as usual. It is also possible that the epinephrine produced in the gland, no matter what the amount may be, is quickly expelled by the abnormal nerve stimulation and consequently the lower concentration soon manifests itself. Quinquaud[5] reported that the epinephrine content of the adrenal was decreased after *piqûre*. Confirming the results of Elliott,[6] Stewart and Rogoff[7] reported that diminution of the stock of epinephrine in the adrenal, through electrical stimulation of the splanchnics, was not easy to demonstrate, despite the fact that the liberation of epinephrine into the blood was notably increased by the stimulation. Only after a stimulation, repeated at intervals for a long time, could a distinct depletion of the epinephrine content be observed. The results in Table II show that the epinephrine content of the adrenal was practically not changed by thyroid feeding (a small dose per day for a long time, and a large dose per day for a short time), neither absolutely nor relatively, the individual or physiological variations being kept in mind. Neither do the absolute and relative weights of the adrenals show any noteworthy difference from those of the control animals. According to Donaldson's tables,[8] the weight of the adrenals of albino rats is 45.6 mg. (average of both sexes) for 200 gm. of body weight, and 48.2 mg. (average of both sexes) for 215 gm. of body weight. In his chart it may be seen that there exists a widely ranging variation. In Herring's report, the epinephrine content of the adrenals of the thyroid-fed animals was increased absolutely but decreased when it was calculated per gm. of the adrenal. The weight of the fresh adrenals

[5] Quinquaud, A., Relations entre la piqûre diabétique et la sécrétion d'adrénaline, Paris, 1915.

[6] Elliott, T. R., *J. Physiol.*, 1912, xliv, 374.

[7] Stewart, G. N., and Rogoff, J. M., *J. Exp. Med.*, 1916, xxiv, 709.

[8] Donaldson, H. H., The Rat, Philadelphia, 1915, 100, 137.

was increased. On the other hand, the desiccated adrenal of the thyroid-fed animals had a higher adrenal content than the same amount of desiccated normal adrenal. This seems to show that the increase of the adrenal weight was mostly due to the water content and not to the solid substance. Obtaining the blood of the adrenal vein of dogs, into which extracts of various organs had been previously injected, and testing the vasoconstrictor action of this blood sample with another dog, Gley and Quinquaud[9] concluded that though the extracts of various organs can augment the secretion of epinephrine, the extract of thyroid gland is not specific. Gley's other experiments[10] demonstrate that the epinephrine content of the adrenal of thyroidectomized dogs and rabbits is not lower than that of normal animals. This is not in harmony with the results of Herring with thyroidectomized cats.[3]

Though an augmented secretion of epinephrine in hyperthyroidism may be probable, the frequently reported intimate relation between the hormones of the thyroid and adrenal has also been studied from different standpoints. Macleod and Pearce[11] observed that after complete dissection of the hepatic plexus, stimulation of the splanchnic nerve is only occasionally followed by an increase in the sugar content of the vena cava blood, although a marked hyperglycemia is found in cases of the intact hepatic plexus. They therefore concluded that there was no evidence that hyperglycemia, caused by stimulation of splanchnic nerve, is due to a hypersecretion of epinephrine. Oswald[12] demonstrated that though intravenous injection of iodothyreoglobulin has no effect on the circulation, this treatment increases the effectiveness of epinephrine in raising and maintaining blood pressure. Asher,[13] Richardson,[14] Kakehi,[14] and Eiger[15] reported that

[9] Gley, E., and Quinquaud, A., *Arch. Internat. Physiol.*, 1914, xiv, 152.

[10] Gley, E., *Arch. Internat. Physiol.*, 1914, xiv, 175.

[11] Macleod, J. J. R., and Pearce, R. G., *Am. J. Physiol.*, 1911–12, xxix, 419.

[12] Oswald, A., *Z. Physiol.*, 1915, xxx, 509; cited by Levy.[16]

[13] Asher, L., *Deutsch. Med. Woch.*, 1916, xxxiv, 1; abstr. in *Physiol. Abst.*, 1916–17, i, 426.

[14] Richardson, H. B., *Z. Biol.*, 1916, lxvii, 57. Kakehi, S., *ibid.*, 1916, lxvii, 104; both papers abstr. in *Physiol. Abst.*, 1916–17, i, 427.

[15] Eiger, M., *Z. Biol.*, 1917, lxvii, 253, 265; abstr. in *Physiol. Abst.*, 1917, ii, 269.

in the experiments with Laewen-Trendelenburg frog or rabbit heart, the action of epinephrine was augmented by the addition of thyroid extract. In the same manner, the plasma of rats fed on thyroid and plasma obtained from patients with Graves' disease, had a well marked accelerating action on vasoconstrictor influence of epinephrine. Asher considers that thyreoglandol, which is protein-free and practically iodine-free, exerts the same biological and metabolic action as the whole gland. Levy[16] reported that in ordinary and adrenalectomized cats stimulation of the cervical sympathetic nerve can increase the effectiveness of epinephrine in raising arterial pressure, although this is not the case in thyroidectomized cats. He concluded that thyroid secretion renders more excitable the sympathetic structures acted on by epinephrine in raising arterial pressure, and the increased effectiveness of epinephrine as a pressor agent after thyroid stimulation is not dependent on a greater amount of circulating epinephrine. On the other hand, however, he and Cannon and Cattell[17] obtained some evidence indicating that stimulation of the cervical sympathetic nerve or epinephrine injection induces secretory activity in the thyroid gland. In my previous experiments,[1] when epinephrine was injected into thyroid-fed rabbits, epinephrine glycosuria was induced to the same extent as in control animals; the hyperglycemia being practically the same or somewhat augmented.

SUMMARY.

The epinephrine content of the adrenals of normal medium sized albino rats is 2.2 mg. per gm. of the gland. Comparing this figure with those reported by other investigators with larger animals, the epinephrine content of rat adrenal is the nearest to that of the dog, larger than that of the cat, and smaller than that of the sheep and calf. Thyroid feeding of either short duration with large doses or long duration with small doses does not materially change the epinephrine content, nor the weight of the adrenals of medium sized albino rats. If hypersecretion of the adrenals really exists in experimental hyperthyroidism, these

[16] Levy, R. L., *Am. J. Physiol.*, 1916, xli, 492.
[17] Cannon, W. B., and Cattell, McK., *Am. J. Physiol.*, 1916, xli, 74.

results indicate that the excess of epinephrine is promptly transported into the circulation, the epinephrine content of the adrenals being kept fairly constant. The presence of such a regulatory mechanism might well be expected from the experiments of Elliott and Stewart and Rogoff.

THE INFLUENCE OF THYROID FEEDING UPON CARBOHYDRATE METABOLISM. III.

THE ACIDOSIS IN EXPERIMENTAL HYPERTHYROIDISM AND ITS RELATION TO EPINEPHRINE IN THE BLOOD AND THE DECREASE OF LIVER GLYCOGEN.

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The Influence of Thyroid Feeding upon the Hydrogen Ion Concentration and the Alkali Reserve of the Blood Plasma.

The influence of acid and alkali upon carbohydrate metabolism, with respect to glycosuria, blood sugar content, and glycogen content of the liver, has been discussed by many investigators. Edie and his coworkers[1] reported that the glycosuria found after partial asphyxiation is due to the high percentage of carbon dioxide in the respired air. MacLeod[2] demonstrated that hyperglycemia and glycosuria in asphyxia and curare poisoning are due to the mobilization of liver glycogen and that here the blood acts directly upon the liver cells without any intermediation of the nervous system. Estimating the rate of disappearance of glycogen from pieces of liver, both in an atmosphere of carbon dioxide and in air or oxygen, he also found that glycogenolysis is more rapid in the former than in the latter. Administering hydrochloric acid by mouth to rabbits and also perfusing turtle livers with a solution acidified with hydrochloric acid, Elias[3] demonstrated that a relatively small amount of acid can mobilize the glycogen of the liver and cause hyperglycemia and glycosuria. Studying the adrenal histologically, he considered that these glands have no direct relations to the glycogen mobilization caused by acid. Elias and Kolb[4] have also concluded that the so called "hunger diabetes" of young dogs, is, in part at least, a condition due to acidosis, as indicated by the lowered carbon dioxide content of the blood and of the alveolar air. The word

[1] Edie, E. S., *Biochem. J.*, 1906, i, 455. Edie, E. S., Moore, B., and Roaf, H. E., *ibid.*, 1911, v, 325.

[2] Macleod, J. J. R., *Am. J. Physiol.*, 1908–09, xxiii, 278.

[3] Elias, H., *Biochem. Z.*, 1913, xlviii, 120.

[4] Elias, H., and Kolb, L., *Biochem. Z.*, 1913, lii, 331.

acidosis is used to express an actual increase in the hydrogen ion concentration of the blood as well as a lowering of the alkali reserve. It has been pointed out that free carbon dioxide is present in the body fluids in such concentration that it automatically converts into bicarbonates all bases not bound by other acids. In acidosis the concentration of bicarbonate in the blood is always reduced below the normal level. As a sensitive indicator of this condition and its severity, the carbon dioxide capacity of the plasma was determined by Van Slyke and Cullen.[5]

Since thyroid feeding can decrease the glycogen content of the liver very easily, possibly acidosis plays some rôle here.

Methods.

Full grown rabbits were used. 3 gm. of desiccated thyroid, suspended in water, were given by a stomach sound every afternoon. Oats or greens (cabbage and carrot tops) were given as food. As shown by Sherman and Gettler,[6] and McDanell and Underhill,[7] the kind of food has a marked influence upon the acid-base equilibrium of the organism. Attention to this was, therefore, paid both in the preparatory and the thyroid period. When greens were given, the thyroid-fed rabbits continued to take them until the later part of the thyroid period in about the same quantities as in the preparatory period. In the case of the oat diet, the appetite of the animal was greatly affected by thyroid feeding. They stopped eating this food in a few days. As control experiments, therefore, rabbits were fasted and others were fed on desiccated animal tissue other than thyroid, no other food being added. As such a tissue powder, a mixture of desiccated spleen, kidney, and parotid gland in equal proportions was used. The amount and the method of administration of this powder were the same as in thyroid feeding. The urine was examined for protein and casts from time to time. The hydrogen ion concentration of the blood was determined by Marriott's method.[8] 1 cc. of oxalated venous blood was used for dialysis. After comparing the color, produced by phenolsulfonephthalein, with the standard colors, the dialysate was

[5] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.
[6] Sherman, H. C., and Gettler, A. O., *J. Biol. Chem.*, 1912, xi, 323.
[7] McDanell, L., and Underhill, F. P., *J. Biol. Chem.*, 1917, xxix, 233.
[8] Marriott, W. McK., *Arch. Int. Med.*, 1916, xvii, 840.

vigorously aerated, and the reaction thus changed, which now served as an index of the alkali reserve of the blood, was again estimated colorimetrically. The CO_2 capacity of the plasma was determined by Van Slyke and Cullen's method,[5] the larger size apparatus[9] being used. The results were calculated so that they showed cc. of CO_2 reduced to 0°C., and 760 mm. Hg, bound as bicarbonate in 100 cc. of the plasma. The blood was collected from the external jugular vein by a needle connecting to a cannula passing to the bottom of a centrifuge tube containing potassium oxalate and a layer of paraffin oil. No anesthetic was used. During the operation the rabbit was kept quiet so as not to disturb the circulation and respiration. 8 or 9 cc. of the venous blood were collected at once. After mixing with the oxalate, part of it was taken out for dialysis and the rest was centrifuged for obtaining the plasma. The same vein could be used for several punctures. But if thrombosis occurred, the vein on the other side was used. The blood was examined usually in the morning. When this was done twice a day, the first examination was made in the morning and the second one in the afternoon after thyroid feeding. On the first experimental day, the blood examination was made before changing foodstuffs or before the first thyroid feeding. The results of this day, therefore, can be considered the normal value. The results are shown in Tables I and II, and Charts I and II.

From these results it will be seen that the hydrogen ion concentration of the normal rabbit blood is pH 7.35 to 7.5. The kind of food caused no noteworthy difference. After aeration of the dialysate the value became pH 8.1 to 8.2. Levy and his coworkers[10] reported that pH of the oxalated blood from normal men varies from 7.4 to 7.6. This is nearly the same as my results with the rabbit blood. When thyroid was given to the oat-fed rabbits, the hydrogen ion concentration of the blood slightly increased (the maximum, pH 7.25 to 7.3). In the blood of acidosis patients, Levy and his coworkers[10] observed a reaction of pH 7.1 to 7.4; therefore nearly the same degree of acidosis as found in my thyroid-fed rabbits. A slight decrease of the

[9] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

[10] Levy, R. L., Rowntree, L. G., and Marriott, W. McK., *Arch. Int. Med.*, 1915, xvi, 389.

TABLE I.

The Influence of Thyroid Feeding upon the Hydrogen Ion Concentration of the Blood and the Alkali Reserve of the Blood Plasma.

Series A. Oats Given Freely.

Experimental day.	Body weight.	Desiccated thyroid.	Oats.	Blood. pH Before aeration.	Blood. pH After aeration.	Blood. Plasma CO_2 capacity.	Urine volume.	Remarks.
Rabbit I. (Before experiment oats only were given freely for 5 days.)								
	kg.	*gm.*	*gm.*				*cc.*	
1	3.06	3	60	7.45	8.1	52.2	116	Urine: protein −, casts −.
2		3	14	7.45	8.1	48.0	130	
3		3	0	7.35	8.0	45.2	100	
4		3	0	7.35	8.0	40.9	106	
5		3	0	7.30 7.25	7.9 7.9	30.5 28.7	52	Urine: protein +, casts +. Died on the 6th day.
Rabbit II. (Before experiment oats only were given freely for 5 days.)								
1	2.48	3	52	7.45	8.1	53.2	93	Urine: protein −, casts −.
2		3	38	7.45	8.1	57.0	80	
3		3	10	7.45	8.1	57.9	65	
4		3	0	7.45	8.1	47.0	69	
5	2.09	0	0	7.25 7.25	8.0 8.0	29.6 29.6	81	Urine: protein ±, casts −.
6		0	Oats and	7.45	8.1	29.6	78	
7		0	greens were	7.45	8.1	43.8	102	
8	2.13	0	given freely.	7.45	8.1	60.7	99	Recovered.
Rabbit III. (Before experiment oats only were given freely for 5 days.)								
1	1.62	3	43	7.35	8.1	51.0	87	Urine: protein −, casts −.
2		3	5	7.35	8.1		151	
3		3	0	7.35	8.1	44.3	131	
4		3	0	7.35	8.1	43.3	126	
5		3	0	7.35	8.1	43.2	92	Urine: protein −, casts −.
6	1.30	3	0	7.35	8.1	37.5	82	
7		0	0	7.3	8.1	29.5	?	Died on 8th day.
Rabbit IV. (Before experiment oats and greens were given freely for 5 days.)								
1	2.10	3	34	7.5	8.2	62.3	154	Urine: protein −, casts −.
2		3	24				140	
3		3	0	7.4	8.2	42.9	?	
4	1.92	3	0				91	Urine: protein ±, casts −.
5		3	0	7.35	8.1	30.3	82	Died on 6th day.

TABLE I—*Concluded.*

Series B. Greens Given Freely.

Experimental day.	Body weight.	Desiccated thyroid.	Blood. pH Before aeration.	Blood. pH After aeration.	Blood. Plasma CO_2 capacity.	Urine volume.	Remarks.
Rabbit I. (Before experiment greens only were given freely for 5 days.)							
	kg.	*gm.*				*cc.*	
1	3.02	3	7.45	8.1	63.6	305	Urine: protein −, casts −.
2		3	7.45	8.2	63.6	410	
3		3	7.45	8.1	60.7	350	
4		3	7.45	8.1	55.3	400	
5		3	7.45	8.1	46.6	292	
6		3	7.45	8.1	48.5	306	
7		3	7.45	8.1	52.2	320	Urine: protein −, casts −.
8	2.20	0	7.45	8.1	43.8	380	Recovered by stopping thyroid feeding.
Rabbit II. (Before experiment greens only were given freely for 3 days.)							
1	1.34	3	7.5	8.1	61.4	320	Urine: protein −, casts −.
2		3				329	
3		3				420	
4		3	7.45	8.1	49.0	266	Urine: protein ±, casts −.
5	0.98	3	7.45	8.1	46.2	408	Died on 6th day.

alkali reserve of the blood was also found in some of the thyroid-fed rabbits, the aerated blood dialysate having a reaction of pH 7.9 to 8.0. When greens were given freely to the thyroid-fed rabbits, such changes in the blood reaction could not be observed. In the control animals, either fasted or fed with animal tissue powder, the hydrogen ion concentration of the blood remained almost unchanged. The kind of food eaten leads to a marked difference in the CO_2 capacity of the blood plasma of the normal rabbits. When greens only were given, the plasma CO_2 capacity was 61.4 to 63.6 (cc. of CO_2, reduced to 0°C., 760 mm. Hg, bound as bicarbonate in 100 cc. of the blood plasma), while this value was reduced to 48.2 to 53.2 on oat diet. When thyroid was given to the animals, the CO_2 capacity of the plasma gradually decreased. On oat diet it went down as far as 28.7

TABLE II.

The Influence of Fasting or Feeding Animal Tissue Powder upon the Hydrogen Ion Concentration of the Blood and the Alkali Reserve of the Blood Plasma.

Experimental day.	Body weight.	Blood. pH Before aeration.	Blood. pH After aeration.	Blood. Plasma CO_2 capacity.	Urine volume.	Remarks.
Rabbit I. Fasting. (Before experiment oats only were given freely for 5 days.)						
	kg.				*cc.*	
1	2.04	7.45	8.1	50.1	60	Urine: protein −, casts −.
2		7.45	8.1	44.5	73	
3					50	
4		7.45	8.1	44.7	48	
5		7.45	8.1	41.9	63	Urine: protein ±, casts −.
6		7.40	8.1	36.0	79	Died on 7th day.
Rabbit II. Fasting. (Before experiment oats and greens were given freely for 5 days.)						
1	2.20	7.5	8.2	62.5	85	Urine: protein −, casts −.
2					133	
3		7.35	8.1	40.0	172	
4					?	
5		7.35	8.1	39.5	150	Urine: protein −, casts −.
6		7.35	8.1	34.7	136	
7		7.35	8.1	34.8	53	
8	1.38					Died.
Rabbit III. Animal tissue powder feeding, 3 gm. per day. No other food. (Before experiment oats only were given freely for 16 days.)						
1	1.78	7.45	8.1	48.2	55	Urine: protein −, casts −.
2					89	
3		7.5	8.1	52.9	153	
4					100	
5		7.4	8.1	44.3	65	
6					73	
7		7.4	8.1	41.3	93	
8					62	Urine: protein −, casts −.
9	1.22	7.4	8.1	42.0		Recovered by giving food.

TABLE II—*Concluded.*

Experimental day.	Body weight.	Blood. pH Before aeration.	Blood. pH After aeration.	Blood. Plasma CO_2 capacity.	Urine volume.	Remarks.
Rabbit IV. Animal tissue powder feeding, 3 gm. per day. No other food. (Before experiment oats and greens were given freely for 7 days.)						
	kg.				*cc.*	
1	1.70	7.4	8.1	55.3	62	Urine: protein −, casts −.
2					55	
3		7.4	8.1	45.0	70	
4					48	
5		7.4	8.1	40.9	59	Urine: protein +, casts −.
6					61	
7	1.40	7.4	8.1	38.5	45	Died 2 days later.

to 30.3, while on green diet the minimum was 43.8 to 46.2. In one case of thyroid-fed rabbits on the oat diet, thyroid feeding was stopped and greens were freely given after the 6th experimental day. The CO_2 capacity of the plasma increased from 29.6 to 60.7 in 2 days. In rabbits either fasted or fed with animal tissue powder, the CO_2 capacity of the plasma was also decreased, the former showing a more marked change than the latter. The minimum of the CO_2 capacity of the plasma was 36.0 to 34.7 (fasted) and 38.5 to 41.3 (animal tissue powder fed).

Judging from these results, it will be seen that in rabbits fed oats, thyroid feeding continued for several days caused a marked degree of acidosis, determined by both hydrogen ion concentration of the blood and the CO_2 capacity of the plasma, this disturbance, however, being reparable to a certain degree by taking a large enough amount of base-forming diet; and that the acidosis of thyroid-fed rabbits is greater than that of fasted or animal tissue powder-fed rabbits. Goto[11] reported that acidosis exists in uranium nephritis. In some of my rabbits the kidneys seemed to be slightly impaired. As this change, if existing at all, was very slight, acidosis observed in my experiments is probably not due to the kidney injury. Acidosis in diabetes is reported to

[11] Goto, K., *J. Exp. Med.*, 1917, xxv, 693.

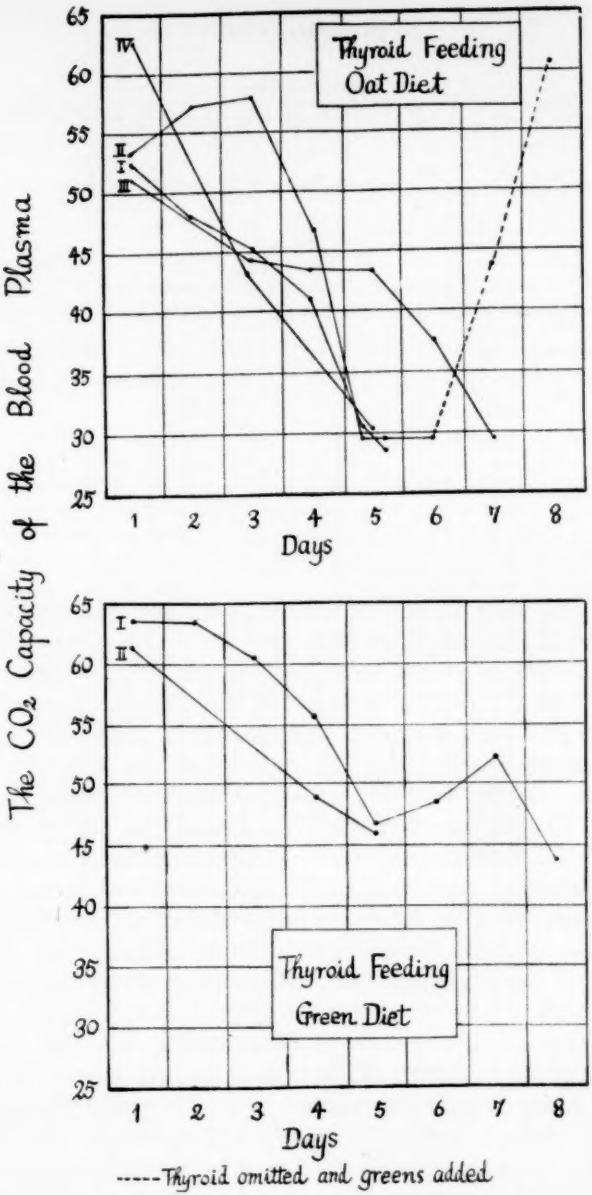


CHART 1. The influence of thyroid feeding upon the CO_2 capacity of the blood plasma.

be due to an overwhelmingly rapid production of acids, and acidosis in nephritis to an inability to eliminate acids which are produced at a moderate rate. Acidosis found in thyroid-fed rabbits is probably due to an abnormal increase of acid production, a shortage of base-forming diet accelerating this acidosis markedly.

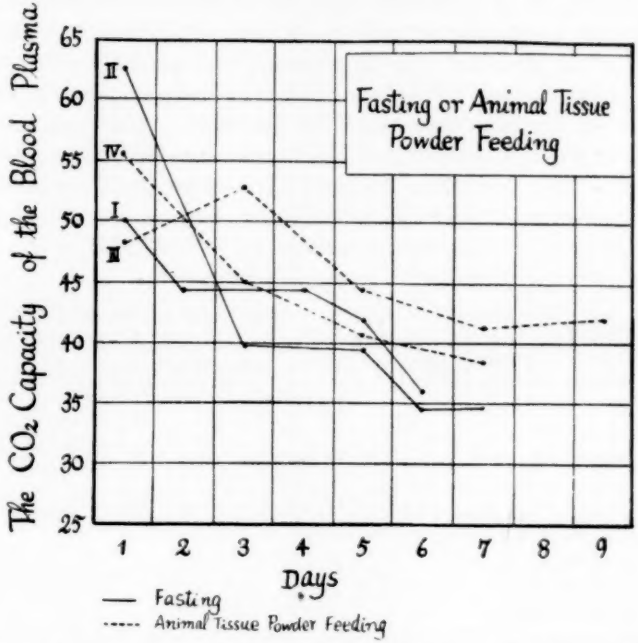


CHART 2. The influence of fasting or of feeding animal tissue powder upon the CO_2 capacity of the blood plasma.

Relations between Epinephrine in the Blood and Acidosis of Thyroid-Fed Rabbits.

Recently Peters and Geyelin[12] reported that subcutaneous injection of epinephrine causes a marked decrease of alkali reserve of the blood plasma, in either diabetic or normal men. This change was parallel with the epinephrine hyperglycemia, the maximum of both being obtained within 3 hours. They considered that at least a part of the hyperglycemia and glycosuria following the injection of epinephrine seems to be caused by a diminution of the alkalinity of the blood.

[12] Peters, J. P., and Geyelin, H. R., *J. Biol. Chem.*, 1917, xxxi, 471.

In order to see whether a diminution of the alkali reserve of the blood after thyroid feeding has any relation to epinephrine, Peters and Geyelin's experiments were repeated on normal rabbits.

Methods.—Adrenalin (1: 1,000 Parke, Davis and Company) was injected subcutaneously into full grown rabbits in doses of 0.5 gm. or 1 mg. of epinephrine per kilo of body weight. Before the experiments some rabbits were fed on oats only, the others on oats and greens for 4 days. Directly before epinephrine injection the animals were fasted for 24 hours. After epinephrine injection the CO_2 capacity of the plasma was determined as described in the preceding chapter. The urine in 24 hours after the injection was collected and its sugar content was determined polariscopically. The details are shown in Table III.

TABLE III.

The Influence of Epinephrine Injection upon the Alkali Reserve of the Blood Plasma. Food in Preparatory Period (4 Days), Nos. III and VI Oats Only; for Others, Oats and Greens.*

Rabbit			I†	II	III	IV	V	VI	VII
Body weight, *kg*			2.38	1.88	1.70	1.30	1.94	1.70	1.72
Plasma CO_2 capacity. (Cc. of CO_2, reduced to 0°, 760 mm., bound as bicarbonate by 100 cc. of plasma.)	Before injection.		54.8	63.2	53.8	53.9	58.6	48.5	56.7
	Hrs. after injection.	½	•			59.5	56.7	50.7	59.5
		1				59.6	55.7	49.2	59.5
		2	61.9	55.6	47.2	60.0			
		4	62.4	59.5	54.4	63.3			
		7	52.8	58.6	54.5				
Urine in 24 hrs. after injection.	Volume, *cc.*		137	103	95	129	116	180	203
	Dextrose, *gm.*		0.81	1.47	0.56	0.42	0.40	0.39	1.79

* Epinephrine injection, 0.5 mg. per kilo of body weight in Rabbits I to VI; 1.0 mg. in No. VII.

† The sugar content of the blood before epinephrine injection, and 2, 4, and 7 hrs. after epinephrine injection respectively was 0.13, 0.33, 0.25, and 0.15 per cent.

From this table it will be seen that in normal rabbits epinephrine injection does not cause any noteworthy decrease of the CO_2 capacity of the plasma. Judging from the glycosuria and hyperglycemia, the drug employed was very effective. The sugar estimation in one case and my previous experiments[13] concerning the epinephrine hyperglycemia show that the maximal point of the epinephrine hyperglycemia is usually obtained from 2 to 4 hours after the subcutaneous injection of epinephrine. No marked decrease of the alkali reserve was observed even at this point. So far as shown by these experiments, no evidence was obtained that hypersecretion of the adrenals plays any rôle in acidosis found in thyroid-fed animals.

The Influence of Sodium Bicarbonate upon the Decrease of the Liver Glycogen of Thyroid-Fed Rats.

Pavy and Bywaters[14] reported that the postmortem production of sugar in the liver may be virtually checked by the injection of a 2 per cent solution of sodium carbonate into the portal system of the living animal. A similar effect of alkali upon the sugar production of the liver was reported by Pavy and Godden[15] in chloroform glycosuria, which is dependent upon the glycogen supply of the liver. A cat was made glycosuric by chloroform anesthesia, and then sodium carbonate, in 3 per cent solution, to the amount of 0.75 gm. per kilo of body weight, was injected into the femoral vein. This brought about a speedy reduction in the glycosuria. Murlin and Kramer[16] reported that in pancreatic diabetes, sodium carbonate decreases glycosuria and that glucose thus retained is not held back as glycogen. According to them, sodium bicarbonate had not the same effect as sodium carbonate. Underhill,[17] however, observed a very favorable effect of sodium bicarbonate in decreasing glycosuria in diabetic patients. Feeding normal rabbits on either base-forming diet (carrot) or acid-forming diet (grain), McDanell and Underhill[18] reported that the former is somewhat more efficient in the formation of liver glycogen than the latter.

In connection with the rapid decrease of the liver glycogen and an onset of acidosis in the thyroid-fed animals, I have made

[13] Kuriyama, S., *J. Biol. Chem.*, 1917, xxix, 127.

[14] Pavy, F. W., and Bywaters, H. W., *J. Physiol.*, 1910–11, xli, 168.

[15] Pavy, F. W., and Godden, W., *J. Physiol.*, 1911–12, xliii, p. vii.

[16] Murlin, J. R., and Kramer, B., *J. Biol. Chem.*, 1916, xxvii, 481. Kramer, B., Marker, J., and Murlin, J. R., *ibid.*, 1916, xxvii, 499.

[17] Underhill, F. P., *J. Am. Med. Assn.*, 1917, lxviii, 497.

[18] McDanell and Underhill, *J. Biol. Chem.*, 1917, xxix, 255.

some experiments on the influence of alkali upon the decrease of the liver glycogen of thyroid-fed animals.

Methods.—Desiccated thyroid was given to albino rats in doses of 1 gm. per day for 4 days. As the main food, dog biscuit-lard paste was used. 0.3 to 0.4 gm. of sodium bicarbonate was mixed with the food every day. When a larger amount of bicarbonate was given, the animal refused to eat it. On the 4th day 1 or 2 gm. of sucrose was given in some cases. In the morning of the 5th day, the liver glycogen was determined. The results are shown in Table IV.

TABLE IV.

The Influence of Sodium Bicarbonate upon the Decrease of Liver Glycogen of Thyroid-Fed Rats.

Rat			I	II	III	IV	V
Body weight, *gm*			255.7	218.6	217.4	209.6	230.1
Desiccated thyroid 1 gm. +	1st day.	Paste, *gm*.	8.0	6.9	6.8	6.6	4.5
		$NaHCO_3$, *gm*.	0.3	0.3	0.4	0.4	0.4
	2nd day.	Paste, *gm*.	6.9	6.6	7.0	6.0	6.0
		$NaHCO_3$, *gm*.	0.3	0.3	0.4	0.4	0.4
	3rd day.	Paste, *gm*.	6.8	6.0	6.2	6.6	6.0
		$NaHCO_3$, *gm*.	0.3	0.3	0.4	0.4	0.4
	4th day.	Paste, *gm*.	6.9	6.8	6.9	6.4	8.0
		Sucrose, *gm*.	0	1.0	0	2.0	0
		$NaHCO_3$, *gm*.	0.3	0.3	0.4	0.4	0.4
		Calories	38.6	42.2	38.6	44.3	44.1
Loss of body weight, *gm*			−8.8	−25.8	−24.0	−21.5	−21.5
Liver.	Weight, *gm*		9.4	6.8	6.3	5.7	6.8
	Glycogen content.	*mg*	0	10.9	Trace.	23.4	0
		per cent.		0.2		0.4	

From this table it will be seen that under the experimental conditions employed, an oral administration of a relatively small amount of sodium bicarbonate does not specially modify the de-

crease of the liver glycogen of thyroid-fed rats. The kind of alkali, the method of administration, and the dose must, however, be taken into consideration. Though it may be possible that the acidosis in experimental hyperthyroidism plays some rôle in restraining the new formation of liver glycogen, further investigations are needed for making this relation clear.

SUMMARY.

In normal rabbits the level of the alkali reserve of the blood is markedly dependent upon the character of the food. Though the CO_2 capacity of the plasma is 61 to 64 in green diet, this value is reduced to 48 to 53 on the oat diet. The hydrogen ion concentration of the blood remains constant with both kinds of diet (pH 7.35 to 7.5). Thyroid feeding induces a noteworthy acidosis in oat-fed rabbits (CO_2 capacity of the plasma, 29 to 30; pH of the blood, 7.25 to 7.3 before aeration, 7.9 to 8.0 after aeration). On green diet these changes are not so marked, probably being repaired by the base-forming character of the food (CO_2 capacity of the plasma, 44 to 46; pH of the blood, 7.45). The acidosis induced by thyroid in oat-fed rabbits can be promptly removed by omitting thyroid from the diet and adding greens. Acidosis appears also in rabbits either fasted or fed with animal tissue powder, but to a smaller degree than in the thyroid-fed animals (CO_2 capacity of the plasma, 36 to 35, and 41 to 39 respectively). Acidosis induced by thyroid feeding is probably due to an increased production of acids in the body.

Epinephrine injection into rabbits failed to cause any noteworthy acidosis. No evidence was obtained that the acidosis found in thyroid-fed rabbits is induced by hypersecretion of the adrenals.

When a small amount of sodium bicarbonate was orally administered into thyroid-fed rats, the decrease of the glycogen content of the liver was nearly as great as that in those without any excess alkali.

The author desires to express his thanks to Professor Frank P. Underhill for his suggestions and interest throughout the course of this and the two foregoing investigations; also to Professor Lafayette B. Mendel for his advice.

THE STRUCTURE OF YEAST NUCLEIC ACID.

II. URIDINEPHOSPHORIC ACID.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATE 1.

(Received for publication, December 1, 1917.)

In a series of articles published in the course of the last few years, Walter Jones[1] and his coworkers advanced a theory on the mode of linkage of the four nucleotides taking part in the molecular structure of yeast nucleic acid. According to these authors, the nucleus of yeast nucleic acid is a tetra-ribose of the following structure $[(C_5H_{10}O_5)_4 - 3\ H_2O]$. Jones and his coworkers have based their conclusions on the analysis of three substances which they had regarded as dinucleotides.

In a previous publication[2] it was pointed out that the theory of Jones and his coworkers was not the only possible conclusion from the facts presented by them. It was further pointed out that not sufficient rigor had been exercised in proving the dinucleotide structure of the substances described by them. In the same publication the present author reported in a preliminary way the results of his own attempts to fractionate the pyrimidine nucleotides described by Levene and Jacobs.[3] For this purpose the crude nucleotides were transformed into the brucine salts and these were repeatedly recrystallized from dry methyl alcohol. In this manner a substance of constant composition was obtained. On conversion of the brucine salt into a barium salt, the composition of the substance was not altered. Both the brucine and the barium salt had the elementary composition of a di-

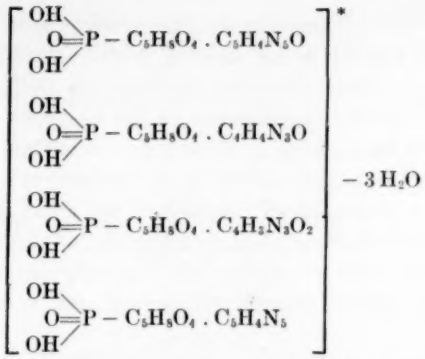
[1] Jones, W., and Richards, A. E., *J. Biol. Chem.*, 1914, xvii, 71. Jones, W., and Germann, H. C., *ibid.*, 1916, xxv, 93. Jones, W., and Read, B. E., *ibid.*, 1917, xxix, 123; xxvi, 39.

[2] Levene, P. A., *J. Biol. Chem.*, 1917, xxxi, 591.

[3] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1911, xliv, 1027; *J. Biol. Chem.*, 1912, xii, 411.

nucleotide. And yet, such a conclusion did not appear entirely compelling, and further attempts at fractionation seemed desirable. Crystallization of the same crude brucine salts from a 35 per cent solution of ethyl alcohol was now resorted to. Passing the salts through eight recrystallizations, it was possible to separate them into two principal fractions. The less soluble part had a composition of the salt of uridinephosphoric acid and the more soluble that of cytidinephosphoric acid. Each of these could be converted into its barium salt. The barium salt of the uridinephosphoric acid precipitated out of a concentrated aqueous solution in the form of long needles, aggregated in rosettes resembling in form those of an osazone. On the other hand, the barium salt of the cytidinephosphoric acid appeared under the same conditions in the form of microscopic granules. The two salts differed in their solubility and in their optical rotation. In this communication only the results of the analysis of uridinephosphoric acid will be reported since attempts are still in progress to obtain the barium salt of the cytidinephosphoric acid also in crystalline form. The purity of the uridinephosphoric acid has been proven by the fact that amino nitrogen could not be detected on the analysis, either of the barium salt directly, or after previous hydrolysis. Nor was it possible to isolate from the product of its hydrolysis any other base than uracil. The specific rotation of the substance was $[\alpha]_D^{20} = +3.5°$.

Thus it is evident that the substance previously described as pyrimidine dinucleotide was a mixture of two mononucleotides. This observation is important inasmuch as it demonstrates conclusively that for the present there is no experimental proof for the assumption of a tetra-ribose as the nucleus of yeast nucleic acid. This, however, does not exclude the possibility that such proof may be furnished in the future. Meanwhile the structural formula of yeast nucleic acid, free from all arbitrary elements, may be written in the following form.



* Bottomley has recently (*Proc. Roy. Soc., B*, 1917, xc, 39) criticized the view expressed by Levene and Medigreceanu (*J. Biol. Chem.*, 1911, ix, 375, 389) that the first phase in the enzymatic cleavage of nucleic acid consisted in the dissolution of the union between individual mononucleotides; Bottomley modified the theory, accepting that the first phase of decomposition of nucleic acid is limited to its cleavage into two dinucleotides. This hypothesis may eventually prove correct; however, the evidence furnished by Bottomley is unsatisfactory. The author made no attempt to fractionate his crude material. For the present the conclusion of Bottomley seems to us unsustained by facts.

EXPERIMENTAL.

The condition of hydrolysis and the method of preparation of the mixed pyrimidine nucleotides were the same as previously described. Care was taken to keep the temperature of the oil bath during the hydrolysis at 100°C.

The crude silver salts were suspended in water and freed from silver by means of hydrogen sulfide. The filtrate from silver sulfide was freed from hydrogen sulfide by aeration, then rendered alkaline by means of a solution of barium hydroxide in order to remove the phosphoric acid. The filtrate was then neutralized and concentrated at diminished pressure and at 50°C. From the concentrated solution the barium was removed quantitatively and to the filtrate brucine in methyl alcoholic solution was added until the originally acid solution turned slightly alkaline to litmus. On standing, a crystalline deposit of the nucleotides formed.

The separation of the two nucleotides was brought about by recrystallization from 35 per cent ethyl alcohol. After eight recrystallizations the brucine salt of a pure uridinenucleotide was

obtained. In the purification of the brucine salt one may, to some extent, be guided by its melting point. The pure material on heating in a capillary tube first contracts at 183°C. (corrected), then melts, and finally decomposes at 198°C. (corrected). However, even after this phase is attained it is advisable to repeat recrystallization at least three times. The optical rotation of the substance was not measured because of its great insolubility in water and in other solvents. The solubility of the salt is greater in dilute alcohol than in water, but the solubility even in this reagent is not great enough to permit an accurate optical measurement.

The composition of the brucine salt (No. 70) was the following:

0.1027 gm. substance gave 0.1998 gm. CO_2 and 0.0584 gm. H_2O.
0.2000 " " " 12.0 cc. of nitrogen at $t° = 22°C.$ and $p = 764$ mm.
0.2000 " " " 0.0168 gm. $Mg_2P_2O_7$.

	Calculated for $C_{10}H_{15}N_2PO_7 + 7\ H_2O$:	Found:
C	53.20	53.06
H	6.51	6.37
N	6.80	6.96
P	2.52	2.37

Conversion of the Brucine Salt into the Barium Salt.—The brucine salt was dissolved in hot water by means of an excess of ammonia water, and the brucine was extracted by means of chloroform in a separating funnel. The aqueous solution of the nucleotide was repeatedly evaporated to dryness under diminished pressure with an excess of barium hydroxide until all ammonium was removed. The residue was then dissolved by the aid of some sulfuric acid, and to the solution a barium hydroxide solution was added until the reaction turned slightly alkaline to phenolphthalein. The filtrate from the barium hydroxide was concentrated under diminished pressure at a temperature of the water bath not exceeding 50°C. It is preferable to interrupt the distillation just before the nucleotide begins to settle out while the distillation is still in progress, since in such case it may have an amorphous appearance. If desired the barium salt may be recrystallized out of water. When air-dry the substance has a granular appearance, and microscopically it consists of rosettes composed of long needles, as is shown in the figures.

Sample 57.

0.1024 gm. substance gave 0.0888 gm. CO_2, 0.0242 gm. H_2O, and 0.0502 gm. $Ba_2P_2O_7$.

0.1015 gm. used for Kjeldahl nitrogen estimation required for neutralization 4.47 cc. 0.1 N acid.

Sample 55.

0.1422 gm. substance used for Kjeldahl nitrogen estimation required for neutralization 6.36 cc. 0.1 N acid.

0.1422 gm. substance gave on fusion 0.0348 gm. $Mg_2P_2O_7$.

0.0711 " " " 0.0356 gm. $BaSO_4$.

	Calculated for $C_9H_{11}N_2O_9PBa$:	Found: No. 55	No. 57
C	23.50		23.65
H	2.41		2.64
N	6.10	6.27	6.16
P	6.75	6.82	
Ba	29.90	29.47	
$Ba_2P_2O_7$	48.97		49.02

The specific rotation of the Ba salt in a 2.5 per cent solution of HCl was

$$[\alpha]_D^{20} = \frac{+0.14 \times 100}{1 \times 4} = +3.5°$$

Hydrolysis of Barium Salt.—9.0 gm. of the barium salt were dissolved in 100.0 cc. of water to which 13.0 gm. of sulfuric acid were added and the solution was heated for 4 hours at 125°C. in a sealed tube. The sulfuric acid was removed quantitatively and the solution was concentrated under diminished pressure until uracil began to crystallize. The mother liquor from uracil was tested for amino nitrogen with a negative result. Also the attempt to prepare an insoluble picrate of cytosine was unsuccessful.

The uracil was recrystallized from dilute sulfuric acid and had the following composition:

0.0984 gm. substance was employed for a Kjeldahl nitrogen estimation and required for neutralization 17.49 cc. of 0.1 N acid.

	Calculated for $C_4H_4N_2O_2$:	Found:
N	25.05	24.88

Cytidinephosphoric Acid.—The mother liquor from the brucine salt of the uridinephosphoric acid was concentrated until a second

crystallization began to deposit. This second brucine salt was converted into a barium salt, which had the composition of the salt of cytidinephosphoric acid. The salt deposited in form of microscopic globules. Attempts are being made to obtain it in a crystalline form and, because of this, the results of the analysis of the substance are deferred to a later date.

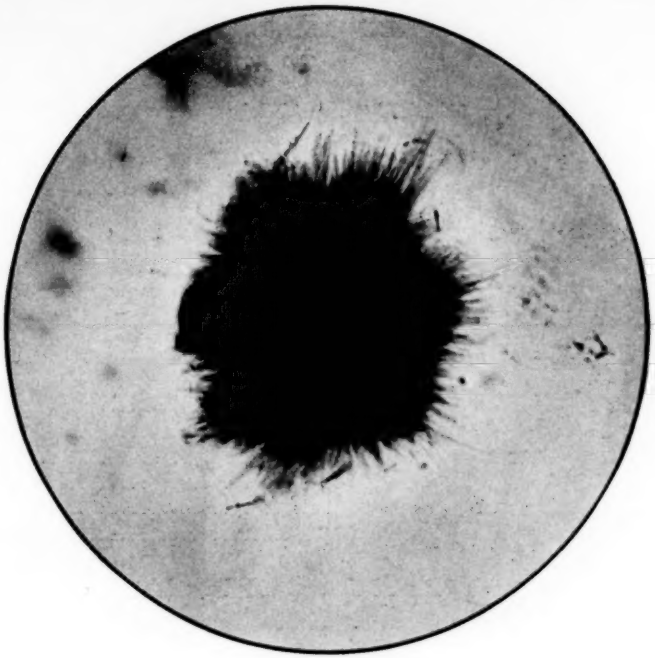


FIG. 1.

FIG. 2.

(Levene: Yeast nucleic acid.)

THE DETERMINATION OF SODIUM AND POTASSIUM

By F. H. McCRUDDEN and C. S. SARGENT.

(*From the Robert B. Brigham Hospital, Boston.*)

(Received for publication, November 26, 1917.)

Sodium and potassium can be separated from other compounds as NaCl + KCl, and the amount of each calculated from the chlorine content of a known weight of the mixture.

$$\begin{aligned} \text{NaCl} + \quad \text{KCl} &= \text{S}^1 \\ \tfrac{35.45}{58.50}\,\text{NaCl} + \tfrac{35.45}{74.56}\,\text{KCl} &= \text{H}^2 \end{aligned}$$

Though the possibilities of error are great, the method is, nevertheless, practicable.

The percentage error in NaCl: percentage error in Cl =

$$\frac{100\,\dfrac{\Delta\text{NaCl}}{\text{NaCl}}}{100\,\dfrac{\Delta\text{H}}{\text{H}}} = \frac{\Delta\text{NaCl}\,.\,\text{H}}{\Delta\text{H}\,.\,\text{NaCl}}$$

(ΔNaCl and ΔH being the absolute errors).

Solving the first two equations:

$$\text{NaCl} = \frac{\text{H} - \frac{35.45}{74.56}\text{S}}{\frac{35.45}{58.50} - \frac{35.45}{74.56}}$$

Differentiating NaCl with respect to H:

$$\frac{\delta\text{NaCl}}{\delta\text{H}} = \frac{1}{\frac{35.45}{58.50} - \frac{35.45}{74.56}}$$

Substituting these values, we get as percentage error in sodium

[1] Sum.

[2] Halide.

$$\frac{1}{\frac{35.45}{58.50}-\frac{35.45}{74.56}} \times \frac{H}{H-\frac{\frac{35.45}{74.56}S}{\frac{35.45}{58.50}-\frac{35.45}{74.56}}} \text{ or } \frac{H}{H-\frac{35.45}{74.56}S}$$

times that in chlorine,[3] a value which increases as the proportion of sodium in the mixture diminishes.

Magnification of Percentage Error in Chlorine $\left(\frac{H}{H-\frac{35.45}{74.56}S}\right)$.

NaCl : KCl	9:1	8:2	7:3	6:4	5:5	4:6	3:7	2:8	1:9
Magnification	5.0	5.3	6.1	7.0	8.1	10.0	13.0	18.4	35.8

The very small error in the McLean-Van Slyke chloride method[4] can abide such ten- or twenty-folding.

The steps in the McLean-Van Slyke method are as follows: (1) add standard silver nitrate to solution of chlorides; (2) make to definite volume, filter, take aliquot part of filtrate; (3) titrate excess of silver nitrate in aliquot part with standard potassium iodide.

The *error in measuring the silver nitrate* with a pipette[5] (experimentally determined) is 0.005 cc. in 20 cc., − 0.025 per cent. (We weighed the irregular amounts of silver nitrate used in our work; the use of a burette at this point would multiply several times the error shown in Table I.)

If no more than 5 per cent excess of silver nitrate is added, the *error in "definite volume and aliquot part"* will be only 5 per cent of the actual error in the measurements (5 per cent of 0.10 per cent = 0.005 per cent).

[3] The total chlorides (NaCl + KCl) can be weighed accurately to 0.1 mg.; but if any error is introduced at this point it will be multiplied $\frac{\frac{35.45}{74.56}S}{H-\frac{35.45}{74.56}S}$ times in the final result.

[4] McLean, F. C., and Van Slyke, D. D., *J. Am. Chem. Soc.*, 1915, xxxvii, 1128.

[5] We used 0.1 N $AgNO_3$ and 0.02 N KI (approximately).

Titration Error.

Experiment No.	Weight of approximately 0.1 N $AgNO_3$.	Approximately 0.02 N KI solution.	KI used for each gm. $AgNO_3$ solution.	Deviation from the mean.
	gm.	cc.	cc.	
1	1.0462	5.75	5.495	0.032
2	1.0492	5.79	5.519	0.008
3	1.0482	5.78	5.514	0.013
4	1.0482	5.79	5.524	0.003
5	1.0489	5.80	5.530	0.003
6	1.0486	5.79	5.522	0.005
7	1.0473	5.82	5.557	0.030
8	1.0473	5.81	5.552	0.025
Average			5.527	0.015

The average deviation from the mean was 0.015 cc.; the extreme deviation only 0.03 cc. If we take 0.04 cc.—the weight of one drop of potassium iodide solution (average of six determinations)—as the titration error, the corresponding error in the (one-fifth as strong[5]) silver nitrate will be (one-fifth of 0.04 cc. =) 0.008 cc. or 0.04 per cent (of 20 cc.).[6]

The error in sodium (0.2 to 0.8 per cent) calculated from a 0.04 per cent error in chlorine is reasonably close to that actually found by experiment (Table I and Chart 1).

Only when five place logarithms are used is the computation error[7] safely less than the analytical error[8] (Table II, Chart 2).

5.8500 gm. of sodium chloride dissolved in water and made up to 1 liter weighed 1,004.1 gm.; 1 gm. of the solution contained 0.0058261 gm. of sodium chloride. 7.4600 gm. potassium chloride dissolved in water and made up to 1 liter weighed 1,005.2 gm.; 1 gm. of the solution contained 0.0074215 gm. of potassium chloride.[9]

[6] Nothing is gained by making the potassium iodide solution either stronger or weaker; with a stronger solution the error is still about one drop—an increase in the absolute error. With a weaker solution, the absolute error increases proportionately; the proportional error remains the same.

[7] The error coming from not preserving enough significant figures in the calculations.

[8] "Four place tables should not be employed upon work of an accuracy exceeding one-half of one per cent," Holman, Computation Rules and Logarithms, New York, 1913, p. lxxvi.

[9] The pure chlorides were made from the purest obtainable commercial products by twice precipitating the salts from saturated aqueous solutions with hydrochloric acid gas. The quantities of these solutions used in all analyses were accurately weighed on the analytical balance.

Chlorine Equivalence of 1 Gm. of Silver Nitrate Solution.

	Against NaCl.	Against KCl.
1	0.0033749	0.0033773
2	0.0033746	0.0033773
3	0.0033754	0.0033746
Average....	0.0033750	0.0033764
Average....	0.0033757	

TABLE I.

Comparison of Calculated and Actual Error.

Parts.		Gm. used.		NaCl found.	Percentage error.		
					Found.		Calculated.
NaCl	KCl	NaCl	KCl			Average.	
		gm.	*gm.*	*gm.*			
8	2	0.10293	0.022813	0.10288	0.05		
		0.10239	0.023311	0.10233	0.06	0.09	0.21
		0.10386	0.023378	0.10370	0.15		
5	5	0.064012	0.067729	0.064155	0.22		
		0.064460	0.067476	0.064699	0.37	0.33	0.32
		0.064327	0.067907	0.064590	0.4		
4	6	0.053420	0.082394	0.053620	0.4	0.23	0.40
		0.052781	0.082571	0.052756	0.05		
3	7	0.041203	0.094061	0.040820	0.9	0.8	0.52
		0.041070	0.093326	0.040780	0.7		
2	8	0.029556	0.11191	0.030209	2.21		
		0.029288	0.11103	0.029673	1.31	1.9	0.74
		0.029487	0.11296	0.030147	2.24		

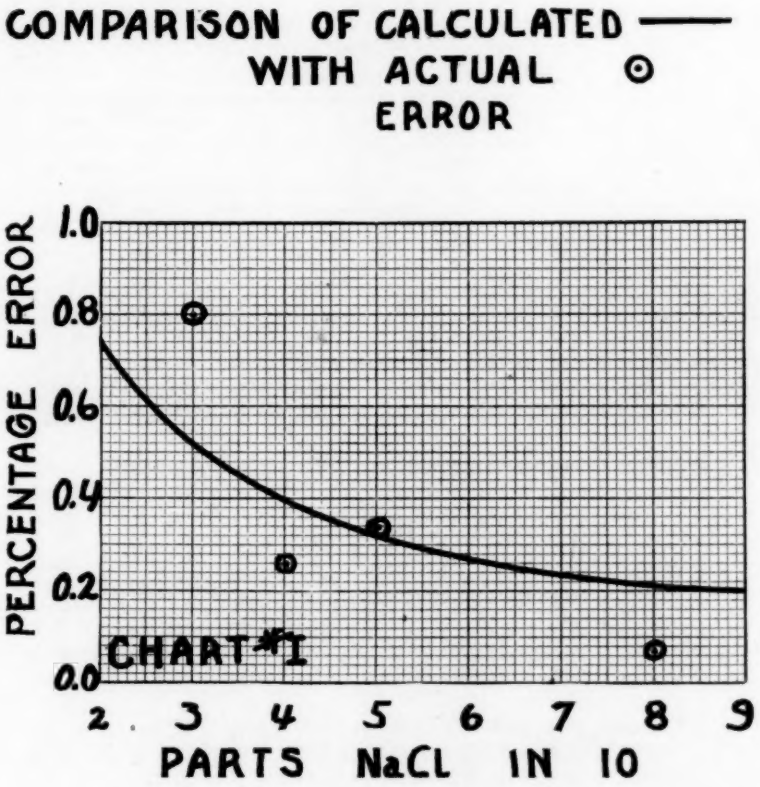


CHART 1. Calculated and actual error. Abscissæ = parts of NaCl in ten parts of NaCl + KCl. Ordinates = percentage error in sodium. Curve = calculated error in sodium for 0.04 per cent error in Cl.

TABLE II.

Comparison of Computation Errors.

Parts.		NaCl					Computation error.					
		Added.	Found.				Slide rule.		Logarithms.			
				Logarithms.					Four place.		Five place.	
NaCl	KCl		12 inch slide rule.	Four place.	Five place.	Six place.		Average.		Average.		Average.
		gm.	*gm.*	*gm.*	*gm.*	*gm.*						
8	2	0.102924	0.1030	0.1030	0.10288	0.102837	0.16		0.16		0.04	
		0.102338	0.1028	0.1025	0.10233	0.102263	0.54	0.3	0.24	0.14	0.07	0.06
		0.103862	0.1034	0.1036	0.10370	0.103623	0.29		0.02		0.07	
5	5	0.064012	0.0650	0.06412	0.064155		1.3		0.05			
		0.064460	0.0650	0.06459	0.064699		0.46	0.5	0.17	0.075		
		0.064327	0.06455	0.06459	0.064590		0.06		0.0			
4	6	0.053420	0.0528	0.05384	0.053620		1.5	0.8	0.4	0.28		
		0.052781	0.0528	0.05284	0.052756		0.1		0.16			
3	7	0.041203	0.0398	0.04076	0.04082		2.4	1.6	0.15	0.28		
		0.041070	0.0405	0.04061	0.04078		0.7		0.4			
2	8	0.0295559	0.03138	0.03038	0.030209	0.0302909	3.6		0.3		0.27	
		0.0292872	0.02984	0.02954	0.029673	0.0297518	0.3	1.7	0.7	0.58	0.27	0.27
		0.0294860	0.03059	0.03000	0.030147	0.0302259	1.2		0.75		0.27	

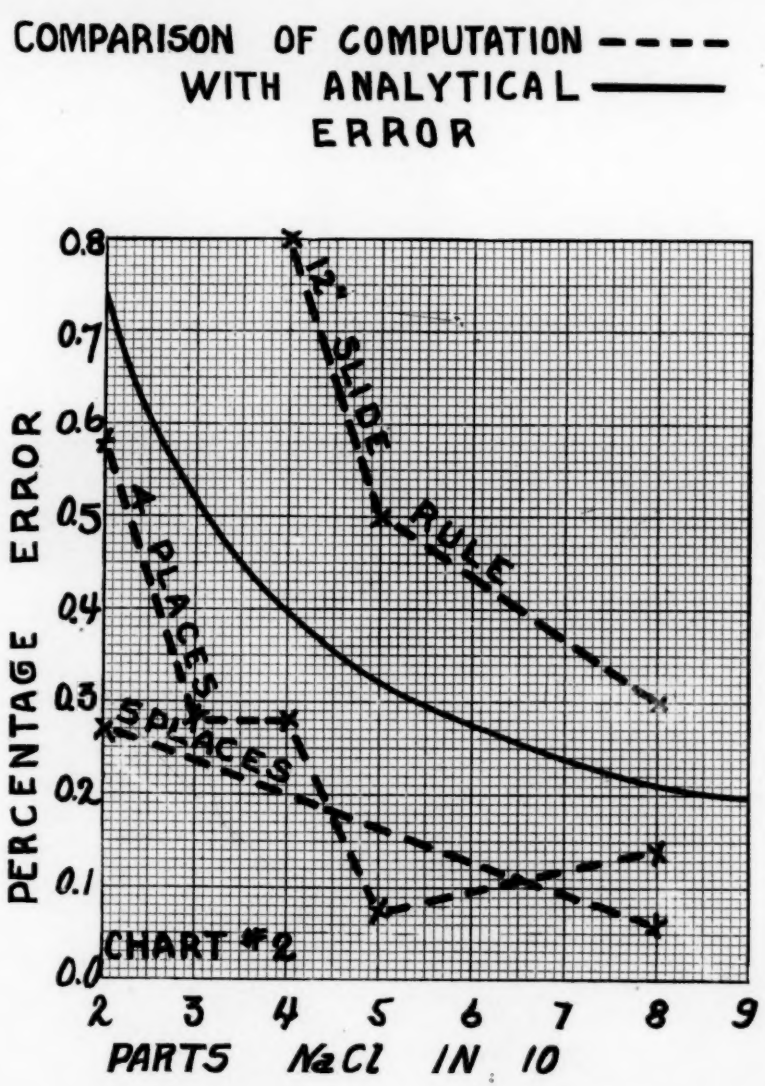


CHART 2. Computation error. Abscissæ = parts of NaCl in ten parts of NaCl + KCl. Ordinates = percentage error in sodium. Full line = analytical error. Dotted lines = computation errors.